CHARACTERIZATION OF PIGMENT AND DISEASE RESISTANCE GENES IN *DENDROBIUM* BREEDING

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ABSTRACT

Dendrobiums are economically important cut flowers and potted plants for production in Hawaii. In 2004 *Dendrobium* was the top selling orchid with a wholesale value of $ 10.5 million. The crop's slow growth, limited available gene pool, and unknown genetics make it very difficult to modify certain desirable traits such as flower color or disease resistance.

The objectives of this research were to characterize pigment and disease resistance genes for use in *Dendrobium* breeding. We demonstrated that the *Dendrobium* dihydroflavonol 4-reductase (*Dfr*) gene has no substrate specificity and is able to utilize all three colorless dihydroflavonoid substrates; namely dihydrokaempferol (DHK), dihydroquercetin (DHQ), and dihydromyricetin (DHM) in order to produce pelargonidin, cyanidin and delphinidin. We were also able to produce several individual plants resistant to *Cymbidium mosaic virus* infection by using transgenesis with a mutated movement protein (*Mut11*). More than half of these plants lack an antibiotic resistance marker gene and thus are particularly desirable for future testing.

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LIST OF ABBREVIATIONS

Enzyme and chemical abbreviations

Anthocyanidin synthase ..... ANS
Chalcone isomerase ..... CHI
Chalcone synthase ..... CHS
Dihydroflavonol 4-reductase ..... DFR
Dihydrokaempferol ..... DHK
Dihydromyricetin ..... DHM
Dihydroquercetin ..... DHQ
Flavonoid 3'-hydroxylase ..... F3'H
Flavonoid 3', 5'-hydroxylase ..... F3'5'H
flavonoid 3 – glucosyltransferase 3GT
Flavanone 3-hydroxylase F3H
β - Glucuronidase ..... GUS
Hygromycin phosphotransferase ..... HPT
Nopalene synthase ..... nos

Other technical terms and abbreviations

Base pairs ..... bp
Cauliflower mosaic virus ..... CaMV
Complementary DNA ..... cDNA
Cymbidium mosaic virus ..... CymMV
Deoxyribonucleotides ..... dNTP
Kilo base pairs (1000 base pairs) ..... kb
KiloDaltons ..... kD
Nanogram (10^{-9} of a gram) ..... ng
Open reading frame ..... ORF
Protocorm-like-bodies ..... PLBs
Polymerase Chain Reaction ..... PCR
Reverse Transcription – Polymease Chain Reaction ..... RT-PCR
Thin Layer Chromatography ..... TLC
Ubiquitin 3 promoter UBQ3

CHAPTER 1

INTRODUCTION

Dendrobium is the most important cut flower orchid in the world. In Hawaii, *Dendrobium* has become the single most valuable commercial flower, given their combined use for cut-flowers, leis, and blooming potted plants (Kamemoto *et al.*, 1999). In 2004, *Dendrobium* was the top selling orchid with a wholesale value of $10.5 million (http://www.nass.usda.gov/hi/flower/dendr.htm/). The genus *Dendrobium* contains a very large number of heterogenous species. It has erect, cane-type pseudobulbs and bears flowers with relatively long vase-life. This group is naturally distributed in warm and humid areas, thus resulting hybrids require warm and humid environment with abundant sunlight for optimum growth and development. The quality of orchid flowers depends on shape, color, vase-life, and no defects from disease and insect damage. For slow-growing orchids, with their long generation times, limited available gene pool, and unknown genetics, it can be difficult to modify certain desirable traits such as flower color or disease resistance through sexual hybridization. Thus genetic engineering coupled with tissue culture techniques is attractive to introduce traits such as improved flower color and disease resistance.

Cymbidium mosaic virus (CyMV) is the most important viral diseases of orchids, resulting in significant losses of yield from the grower. It causes chlorotic to necrotic sunken patches on leaves, necrosis on flowers, and reduces plant vigor, growth rate, and

flower quality although asymptomatic plants are also common (Lawson and Brannigan, 1986; Hu *et al.,* 1993). This virus is spread by use of contaminated tools during propagation of plants and flower harvest (Hu *et al.*, 1994), resulting in difficult diagnoses and control of spread of the virus. It also systemically infects all parts of the orchid plant, including leaves, flowers, pollen, roots, and epidermal cells (Yuen *et al.*, 1979).

Flower color is an important trait for the consumer. Classical breeding techniques have given rise to many commercially successful hybrids with attractive flower colors and forms combined with long vase life, fragrance, seasonality and desirable spray length. However, some colors such as orange-red and blue are missing from *Dendrobium* flower color spectrum (Kuehnle *et al.*, 1997). Genetic modification of pigment biosynthesis through biotechnology is becoming an integral part of breeding new colors in ornamental plants (Davies *et al.,* 2003). One transgenic ornamental, color-modified carnations, is already available in the market and is well received by consumers. The study of pigment biosynthesis pathway is important in developing strategies to modify flower color. Taken together, an understanding of the chemical, histological and molecular aspects of flower color and a potential gene for resistance to CymMV in *Dendrobium* are crucial for the development of breeding strategies to meet grower and consumer needs.

Thus, the objectives of this study were to assess the substrate specificity of *Dendrobium Dfr* gene and to determine the virus resistant properties of *Dendrobium* plants, which contain a CymMV cDNA clone encoding for a movement protein with a site-specific mutation (*Mut11*). Results show that *Dendrobium* DFR enzyme does not

have substrate specificity and efficiently catalyzes the reduction of DHK, DHQ and DHM resulting in production of pelargonidin, cyanidin and delphinidin pigments.

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CHAPTER 2

REVIEW OF LITERATURE

2.1 BRIEF OVERVIEW OF ORCHIDS

The orchids are among the most advanced of flowering plants, highly specialized in many ways. There are about 750 genera with 18,000 species and the Orchidaceae is among the largest families of the flowering plants (Dressler, 1993). The genus *Dendrobium* contains the largest diversity of horticulturally interesting specimens. There are more than 1,000 species in genus *Dendrobium* make it the second-largest orchid genus, next to *Bulbophyllum* (Arditti, 1992). This group is naturally distributed in tropical area, resulting hybrids require warm and humid environment with plenty sunlight for optimum growth and development. Most dendrobiums are epiphytes according to their growth habits, grow on the bark of other plants, but are not parasites. The variation in color and form of flower is extensive and the flowers mimic bees, wasps, butterflies, moths among others (Dunsterville, 1964). This is the result of coevolution with pollen vectors and insect-specific pollination, including pseudocopulation. Orchids are out-breeders and produce an enormous amount of seed (Khoshoo, 1986). Orchids have very small seeds without endosperm. Thus they require a mycorrhizal association for their germination (Cherevchenke and Bohalyr, 1984). The associations of mycorrhiza, usually of species *Rhizoctonia* are believed to provide simple sugars and other nutrients needed for seed germination (Arditti, 1967).

Orchid flowers are different from other monocot flowers make them very distinguish among the other. The flowers compose of three similar sepals and petals same as other monocots. The difference is one of the sepals is modified into lip structure called labellum. The filaments, anthers, style and stigma are reduced and fused into a single structure called the column. The inner side of the column is the stigma and the top of column is group of anther called pollinia.

In 1856, the first manmade orchid flowered was established and 34 years later, in orchid hybrids became available in 1890. Later on, an American botanist, L. Knudson, developed a method of propagating plants from seed by culturing in sterile flasks containing agar and sugar in 1922. This was very important milestone in orchid culture since it was showed the possibility to culture orchids without association with mycorrhiza. In 1956, G.M. Morel discovered a method of mericloning, which opened large possibilities of multiplying orchid hybrids. Rapid and large-scale clonal propagation of orchids is made possible by using the batch tissue culture procedure. At least 43 orchid genera have been cloned successfully using different plant parts including apical meristem, flower stalks, axillary buds, leaves, and roots (Arditti and Ernst, 1993).

Generally, orchid seedlings that are grown in flasks are first transferred to a community pot, then to two inch (in diameter) pots, after that to a 4 inch (in diameter) pots, and finally to a 6 inch (in diameter) pots. The duration for each transfer is about three to six months. In the tropics, it may take more than two years for the orchid plantlets to reach the flowering stage (Hew and Yong, 1997). In Hawaii, *Dendrobium* hybrids' seeds were germinated *in vitro* around three months after pollination and were

transflasked three months later. Seedlings were transplanted into community pots (six inches clay pot contained basaltic gravel (blue rock) size 1.5 to 2.25 inches (size 3) on the bottom and chopped hapuu on the top. Nutricote (13-13-13) + 100 days release was used as fertilizer at this stage. Six months later, the seedlings were moved from community pots to 2-inch pots using only hapuu as a medium. After 9 to ten months in 2-inch pots, seedlings were moved to 6-inch black plastic pots using blue rock (No. 3) as a medium. Normally, the first flower will be obtained about two and a half to three years after seeds germination (Kamemoto *et al.*, 1989).

One of the important aspects of orchid industry is hybridization. There have to be specific objectives for raising hybrids, like robust, colorful, longer lasting flowers and disease resistance.

2.2 DISEASES THAT CAUSE FLORAL AND FOLIAR SYMPTOMS SIMILAR TO VIRUS IN *DENDROBIUM* ORCHIDS

There are several diseases cause by bacteria and fungus that induce floral and foliar symptoms as well as virus infection as describe in table 2.1.

Table 2.1 Diseases that cause floral and foliar symptoms similar to virus in *Dendrobium* orchids.

Pathogens	Symptom	References
Botrytis cinerea	circular and brown or pink spots on orchid flowers	Uchida, 1994
Collectotrichum species	leaf and blossom spots	Uchida, 1994
Phyllosticta capitalensis	Circular yellow spots on leaves, reduce yield	Uchida, 1994
Fusarium proliferatum	oval and dark brown spots on flowers and brown to blackish-brown spot on leaves	Uchida, 1994
Phytophthora	olive-green to greenish yellow and darken to brown leaf spots, reduces plant size and vigor	Uchida and Aragaki, 1991
Erwinia chrysanthemi	Leaf spots, the surrounding tissue may be yellow, while the center of the spot becomes tan to brown.	Keith *et al.*, 2003
Pseudomonas gladioli pv. *gladioli*	Leaf spots, the surrounding tissue may be yellow, while the center of the spot becomes tan to brown.	Keith *et al.*, 2003

2.3 *CYMBIDIUM MOSAIC VIRUS* IN *DENDROBIUM* ORCHIDS

2.3.1 Disease caused by *Cymbidium Mosaic Virus* virus

Some viruses replicate within the plant and are usually systemic within the plant. Thus, for plants such as orchids that are vegetatively reproduced, the virus can be a permanent part of the plant and multiply within it. Since 1952, *Cymbidium Mosaic Virus* (CymMV) has been recognized as the most economically important virus that affects not only the vigor of orchid plants but also decreases the quality of flowers produced by the diseased plants (Faccioli and Marani, 1979; Ajjikuttira *et al.*, 2005). Symptoms of CymMV infection include brown streaks on the lips of white *Dendrobium* cultivars, brown streaks and color breaking on petals and chlorotic to necrosis sunken patch on leaves. Leaf symptoms are common on the under-surface, but both surfaces can have chlorotic or necrosis lesions. The infection of CymMV results in growth reduction, decreased plant vigor and flower yield. Infected plants may not show disease symptoms or symptomless (Dunn, 1980).

The virus systemically infects all parts of the orchid plants, including leaves, flowers, pollens, roots, and epidermal cells (Yuen *et al.*, 1979) and seem to be generally restricted to the family Orchidaceae. CymMV spreads easily by mechanical transmission during harvesting of flowers, and dividing of plants by infected tools and pots (Hathaway, 1991; Hu *et al.*, 1994). Expression of floral necrosis appears to be genetically controlled, with expression being dominant to no-expression depend on the interaction between the host, the pathogen, and the environment (Carrington *et al.*, 1996). Breeding efforts for resistance have proven unfruitful (Kuehnle, 1996). CymMV can be

transmitted by sap inoculation to other families such as *Cassia occidentalis* (L) (Caesalpiniaceae), *Chenopodium amaranticolor* (L) (Chenopodiaceae), and *Datura stramonium* (L) (Solanaceae) (Francki, 1970). In nurseries only four percent of plant samples were positive for CymMV when the plants were less than three years old. The percentage of infection with CymMV increased linearly about 15 % per year for plants 4 to 7 years old and being 100 % infected with 9 to 10 years old plants (Okemura *et al.*, 1984). CymMV was highly heat stable and remained infectious in orchid sap after 10 minutes at 65 °C or 7 days at room temperature (Okemura *et al.*, 1984).

CymMV, a member of the potex virus family, was described by Jensen over 50 years ago (Jensen, 1951). CymMV is a flexuous rod belonging to the potexvirus group (Zettler *et al.*, 1990). The potexviruses form a large group of flexuous filamentous plant viruses with modal lengths between 470 and 850 nm (Wong *et al.*, 1997). CymMV is a single-stranded RNA in a protein coat (Hathaway, 1991), and its size is 415 – 417 nm x 13 – 18 nm (Lawson and Brannigan, 1986). CymMV contains about 6 – 7 kb positive-sense genomic RNA capped and polyadenylated (Francki *et al.*, 1985). Purified CymMV RNA has a length of 6.8 kb. *In vitro* translation of the RNA resulted in the synthesis of eight polypeptides of the following sizes: 74, 60, 50, 37, 26, 17, 11 and 4 kDa (Steinhart and Oshiro, 1990).

The genome organization of the potexviruses was generally well conserved and composed of coding sequences for a putative RNA-dependent RNA polymerase (RdRp), a triple-gene-block (TGB), and a coat protein (Fig. 4.1A). With the exception of open reading frame (ORF) 4, all ORFs of potexviruses encode proteins containing consensus

motifs (Wong *et al.*, 1997). The nucleotide sequence of the coat protein gene and two of the TGB genes of different CymMV isolates have been reported (Chia *et al.*, 1992; Neo *et al.*, 1992; Neo *et al.*, 1993; Ryu *et al.*, 1995; Youn *et al.*, 1997; Wong *et al.*, 1998). A complete nucleotide sequence of the genomic RNA of CymMV was 6277 nt in length, excluding the polyadenylated region at the 3′ terminus. Similar to other potexviruses, its genome organization is composed of five major ORFs (ORFs1 to 5), encoding a M_r 26 KDa / 13 KDa / 10 KDa TGB and a M_r 24 KDa coat protein. Comparison among encoded proteins of CymMV and those of other viruses of the potexvirus group revealed that they shared a high degree of homology. All of them have the nucleotide sequence of 5′ non-coding region (NCR) starts with GAAAA, but CymMV possesses the shortest 5′ NCR among all potexviruses. Based on phylogenetic comparisons of RdRp and coat protein, CymMV shares a close relationship to potato aucuba mosaic virus (PAMV), narcissus mosaic virus (NMV), white clover mosaic potex virus (WClMV), and strawberry mild yellow edge-associated virus (SMYEaV) (Wong *et al.*, 1997).

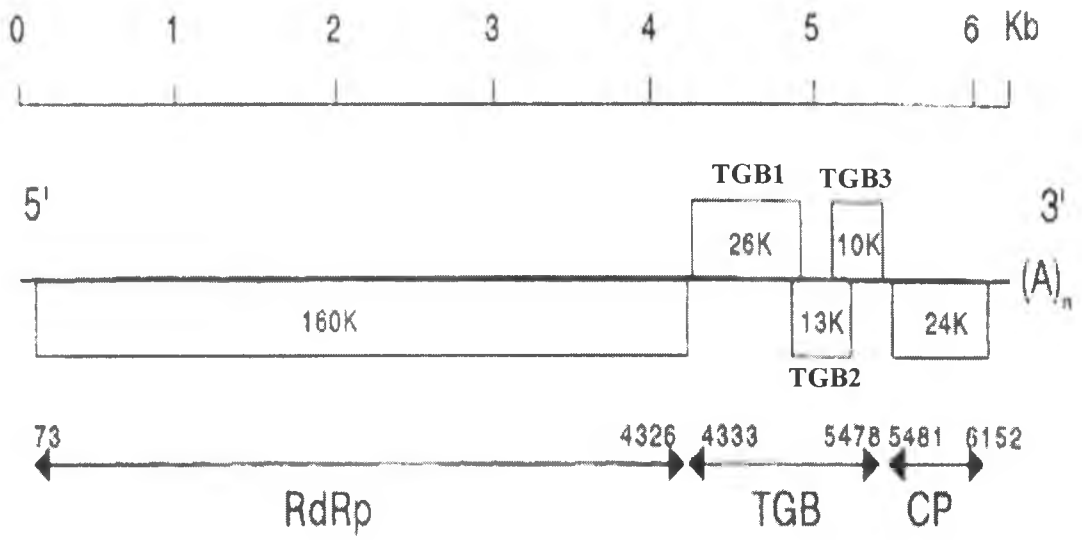

Figure 2.1 Schematic representation of the genome of CymMV. Genome organization with the scale. Open boxes represent the coding regions for the RNA-dependent RNA polymerase (*RdRp*) or 160 Kda, 26 KDa/ 13 KDa/ 10KDa triple gene block (*TGB*) 1, 2 & 3 and 24 KDa coat protein (*CP*). The 5' and 3' noncoding regions are represented as single line. The $(A)_n$ represent the poly (A) tail. Numericals represent nucleotide positions (modified from Wong, S.M., P.H. Mahtani, K.C. Lee, H.H. Yu, Y. Tan, K.K. Neo, Y. Chan, M. Wu, and C.G. Chng. 1997. Cymbidium mosaic potexvirus RNA: complete nucleotide sequence and phylogenetic analysis. Arch. Virology. 142: 383 – 391).

2.3.2 Method for Controlling CymMV

CymMV is not transmitted by natural vectors but is spread by contaminated tools and pots during separation of plants for asexual propagation and harvest of flowers (Hu *et al.*, 1994). The effective ways to control the virus spread include burning of orchid plants known to contain virus, separation of suspected diseased plants, preventing water splash from diseased plants onto another plants close to them, using new sterile media when repotting, minimizing handling of plants, and decontaminating any surface that may have come into contact with the plant sap (Hathaway, 1991; Lawson and Brannigan, 1986; Wisler, 1989). The recommened control methods for CymMV involve sanitation practices and the use of chemicals to sterilize pruning tools (Lawson and Brannigen, 1986; Wisler, 1989, Hu *et al.*, 1994). Sodium hypochlorite, skim milk, ethanol, Agribrom, and Physan have been used by orchid growers in Hawaii to inactivate the viruses on cutting tools (Hu *et al.*, 1994). Several methods provide virus-free materials, but plants remained susceptible to virus (Kuehnle, 1996). Chemotherapy using the antiviral compounds dithiouracil or ribavirin was found suitable for the recovery of CymMV negative tissue and subsequent normal plant development from protocorm-like bodies that were originally virus infected. A genetic alternative to chemotherapy, androgenesis coupled with chromosome doubling by colchicine treatment, was reported effective in ridding amphidiploid stud plants of virus (Kuehnle, 1996).

For genetic engineering of resistance, a CymMV *CP* gene was placed downstream of a cauliflower mosaic virus 35S promoter and the chimaeric gene was transferred into *Nicotiana benthamiana*. Transgenic plants expressing the CymMV *CP* were protected

against CymMV infection. CymMV is capable to replicate on inoculated leaves, but there is no systemic infection (Chia, 1992). Lim *et al.* (1999) introduced the full-length antisense CymMV *CP* gene into *N. occidentalis,* a CymMV systemic propagation host and cymbidium orchid. It delayed symptom appearance, reduction of symptom severity, and tolerance to systemic infection in *N. occidentalis,* but there is no result from transgenic cymbidium yet according to the slow growing of this orchid. Liao *et al.* (2004) transformed a DNA cassette containing a CymMV coat protein (*CP*) cDNA and a nos terminator placed downstream of a maize ubiquitin promoter into *Phalaenopsis* by using particle bombardment. The result showed that five among the 13 tested lines showed CymMV protection in more than 50 % of their progenies 90 day post inoculation (psi) CymMV-inoculation. However, the *CP* transcripts were not detected by northern blot analysis in those progenies, but showed positive results by PCR analysis. These results indicated that the transgene was still present in the genome of those progenies. These results suggested that a RNA-mediated mechanism might be occurred and conferred the resistance to CymMV. Nuclear run on was performed on those resistant plants and siRNAs were detected. This study indicated that *Phalaenopsis* expressing CymMV *CP* effectively confer resistance to CymMV via RNA-mediated through a post-transcriptional gene silencing mechanism (Liao *et al.*, 2004). Chang *et al.* (2005) also used the full-length of capsid protein (CP) gene transformed into *Dendrobium* x Hickam Deb. The results showed that transgenic *Dendrobiums* expressed low level of CymMV compared to the wild type control 120 days post inoculation (Chang *et al.*, 2005). Abundant coat protein in transgenic plants might interfere with the virus uncoating step, which is a prerequisite to the replication of the viral genome after infection (Goldbach *et*

al., 2003). Borth *et al.*, (in press) found that a coat protein strategy was effective in *N. benthamiana*. Four of sixteen (25 %) transgenic *N. benthamiana* lines transformed with the *CP* gene were resistant to CymMV. Using the same *CP* coding sequence, but *CP* transgene into *Dendrobium* remained susceptible.

The improvement of virus resistance in crop plants by means of genetic engineering is a very promising approach. Several strategies for virus resistance engineering have also been tested in plants (Table 2.2).

Table 2.2 Genetic engineering for virus resistance by using different gene strategies.

Gene Express	Target Virus/Group	Transgenic Plant	Resistance Mechanism	Resistance Result	Reference
Coat Protein					
CymMV CP	CymMV/Potexvirus	*Phalaenopsis* orchid 'TS97K' (*P.* x Amabilis W1-10 X *P.* x Amabilis W1-22)	PTGS	Completely Resistance 90 dpi	Chan *et al.*, 2005
CymMV CP	CymMV/Potexvirus	*Phalaenopsis* orchid 'TS340' (*P.* x Taisuco Kochdiam X *P.* x Taisuco Kaaladian)	PTGS	Completely Resistance 90 dpi	Liao *et al.*, 2004
SqMV CP	SqMV/Comovirus	Squash plants	PTGS	Completely Resistance	Pang *et al.*, 2000
PRSV CP	PRSV/Potyvirus	Papaya (*Carica papaya* L.)	PTGS	Completely Resistance	Gonsalves, 2002
TuMV	TuMV/Potyvirus	Oilseed rape (*Brassica napus* cv. Westar)	CP-mediated resistance	Different level of resistance	Lehmann *et al.*, 2003

Table 2.2 Genetic engineering for virus resistance by using different gene strategies (continued).

Gene Express	Target Virus/Group	Transgenic Plant	Resistance Mechanism	Resistance Result	Reference
Coat Protein					
CymMV CP	CymMV/Potexvirus	*Dendrobium* x Hickam Deb	CP-mediated resistance	Lower expression of CymMV in transgenic plants compared to WT 120 dpi	Chang *et al.*, 2005
CymMV CP	CymMV/Potexvirus	*N. benthamiana*	CP-mediated resistance	High titer on inoculated leaves, but no systemic infection to all over the plants	Chia *et al.*, 1992
CymMV antisense CP	CymMV/Potexvirus	*N. occidentalis*	Antisense CP-mediated resistance	Lower expression of CymMV in transgenic plants compared to WT	Lim *et al.*, 1999

Table 2.2 Genetic engineering for virus resistance by using different gene strategies (continued).

Gene Express	Target Virus/Group	Transgenic Plant	Resistance Mechanism	Resistance Result	Reference
Coat Protein					
CymMV CP	CymMV/Potexvirus	*N. benthamiana* and *Dendrobium* orchid	CP-mediated resistance	25% of *N. benthamiana* were resistant to CymMV, whereas non of transgenic *Dendrobium* orchids were resistant	Borth *et al.*, (in press)
Movement Protein					
Mutated CymMV MP	CymMV/Potexvirus	*N. benthamiana*	Mutated MP-mediated resistance	33% of *N. benthamiana* were resistant to CymMV	Borth *et al.*, (in press)

Table 2.2 Genetic engineering for virus resistance by using different gene strategies (continued).

Gene Express	Target Virus/Group	Transgenic Plant	Resistance Mechanism	Resistance Result	Reference
Movement Protein					
Defective TMV MP	TMV/Tobamovirus, TRSV/Nepovirus, PClSV/Caulimovirus, AlMV/Alfamovirus, CMV/ Cucumovirus	*N. tabacum* cv. Xanthi NN	Defective MP-mediated resistance	Resistance to TMV, delay symtomp to TRSV, PClSV, AlMV, CMV	Cooper *et al.*, 1995
Mutant PVX MP	PVX, PMAV/Potexvirus, PVM, PVS/ Carlavirus	Potato (*Solanum tuberosum* cv. Pito)	Mutated MP-mediated resistance	Resistance to PVX, PMAV, PVM, PVS	Seppanen *et al.*, 1997
Others					
PVX Replicase	PVX/Potexvirus	*N. tabacum* (cv. Samsun nn)	Replicase-mediated resistance	Resistance to PVX	Braun *et al.*, 1992

Table 2.2 Genetic engineering for virus resistance by using different gene strategies (continued).

Gene Express	Target Virus/Group	Transgenic Plant	Resistance Mechanism	Resistance Result	Reference
Others					
PVY Replicase	PVY/Potyvirus	*N. tabacum* (cv. Turkish Samsun NN)	Replicase-mediated resistance	Resistance to PVY	Audy *et al.*, 1994
BYDV 5′ half of the BYDV strain PAV genome	BYDV/ Luteovirus	Oat	unknown	Resistance to BYDV	Koev *et al.*, 1998
PLRV Replicase	PLRV/Luteovirus	Russet Burbank Potato	Replicase-mediated resistance	Resistance to PLRV	Lawson *et al.*, 2001

Table 2.2 Genetic engineering for virus resistance by using different gene strategies (continued).

Gene Express	Target Virus/Group	Transgenic Plant	Resistance Mechanism	Resistance Result	Reference
Others					
PVY hpRNA	PVY/Potyvirus	*N. tabacum*	PTGS	Complete Resistance	Smith *et al.*, 2000
PPY *Ihp rolC* gene (*i*ntron *h*air *p*in *rolC*)	PPY/Potyvirus	*N. benthamiana*	PTGS	Complete Resistance	Pandolfini *et al.*, 2003

Virus Abbreviation

CymMV = *Cymbidium mosaic virus,* SqMV = *Squash mosaic como virus,* PRSV = *Papaya ringspot virus* TuMV = *Turnip mosaic virus,* WClMV strain 'O' = *White clover mosaic virus*, TMV = *Tobacco mosaic tobamovirus* PVX = *Potato virus* X, PAMV = *Potato aucuba mosaic potexvirus*, PVM = *Potato virus* M, PVS = *Potato virus* S BYDV = *Barley yellow dwarf virus,* PLRV = *Potato leafroll polerovirus.* TRSV = *Tobacco ringspot nepovirus* PClSV = *Peanut chlorotic streak caulimovirus*, AlMV = *Alfalfa mosaic alfamovirus*, CMV = *Cucumber mosaic Cucumovirus.*

2.3.3 Virus Resistance Genetic Engineering Using Movement Protein Gene

The mechanism for viruses to infect plants, they have to translocation from the infection site to the surrounding cells and over long distances by using the pre-existing pathways within the plants (Ajjikuttira *et al.*, 2005). Virus-encoded proteins facilitate the movement of viruses through plants. For cell-to-cell translocation of viral genomes, many (if not all) plant viruses express "movement proteins" (MPs) which responsible for the spread of virus to other plant tissues and coat protein (CPs), in addition to encapsidating viral RNA, have been shown to facilitate systemic movement in some viruses (Carrington *et al.*, 1996). These movement proteins will interact with endogenous plasmodesmal proteins to increase the plasmodesmal size exclusion limit (SEL) when expressed in transgenic plants (Lucas *et al.*, 1996).

The non-virion 30 kDa protein of TMV was the first specific viral protein identified that could support intercellular plant virus spread (Leonard and Zaitlin, 1982; Ohno *et al.*, 1983; Doem *et al.*, 1987) and, therefore, was defined as a movement protein (MP) (Hull, 1989; Atabekov and Taliansky, 1990).

There are three evidences that supported this hypothesis by studying the movement of *Tobacco mosaic virus* (TMV) in transgenic tobacco. It has been showed that the movement protein may be responsible for plasmodesmata alteration. (i) The TMV MP alters the molecular size exclusion of plasmodesmata in transgenic plant (Wolf *et al.*, 1989). In these studies, the results showed the detection of movement of MP conjugated with fluorescein isothiocyanate-dextran (FITC-dextran), which has a molecular mass of 9.4 kDa in leaf mesophyll of plant expressing MP gene compared to

control plants that have the size exclusion limit of 0.7 to 0.8 kDa. (ii) Labeling the TMV with immunogold showed MP is localized in both cell wall and plasmodesmata (Tomenius *et al.,* 1987). (iii). The MP from TMV is expressed in the cell wall of transgenic plants expressing the MP which is also found in the virus-infected plants (Doem *et al.*, 1987).

The genomes of the potexviruses, carlaviruses, hordeiviruses and some furoviruses are different from TMV and many other viruses having a single movement protein gene according to comparisons of genomic sequences revealed a similar element of three partially overlapping ORFs called the "Triple gene block" (TGB) (Morozov *et al.*, 1989; Foster *et al.*, 1988; Huisman *et al.*, 1988; Skryabin *et al.*, 1988; Rupasov *et al.*, 1989). Each TGB is produced different proteins and TGB-encoded proteins are referred to as TGBp1, TGBp2, and TGBp3, according to the positions of their gene (Solovyev *et al.*, 1996). Mutational analyses of infectious cDNA clones of virus genomes indicated that all three TGB proteins are essential for the virus movement process (Petty and Jackson, 1990; Beck *et al.*, 1991; Gilmer *et al.*, 1992; Herzog *et al.*, 1998). Thus, three proteins in TGB-containing viruses are likely to be carried on the same movement function with the single TMV MP.

Lapidot *et al.* (1993) used a dysfunctional movement protein (dMP) of tobacco mosaic tobamovirus (TMV) caused by deletion of three amino acids. The result showed that the transgenic *Nicotiana tabacum* cvs. Xanthi nn and Xanthi NN that carried the dMP delayed the development of systemic infection to TMV and a number of other tobamoviruses. The delay of systemic infection or partial resistance caused a restriction

in numbers of multicellular infection sites, and a reduction in the rate of local and systemic spread of virus infection (Lapidot *et al.*, 1993). Further work with these tobacco plants by Cooper *et al.* (1995) showed that the dMP of TMV conferred the resistance to TMV and delayed systemic infection against a number of non-tobamoviruses, including alfafa mosaic alfamovirus (AlMV), peanut chlorotic streak caulimovirus (PClSV), tobacco rattle tobravirus (TRV), tobacco ringspot nepovirus (TRSV), and potato virus Y potyvirus (PVY). The dMP restricted the local spread of the tobamoviruses but it did not do so in non-tobamoviruses. The heterologous viruses tested were capable of infection and local spread but were restricted in movement from the inoculated leaf to upper non-inoculated leaves and significant reduction in the development of systemic disease symptoms (Cooper *et al.*, 1995).

Beck *et al.* (1994) described a mutation in p13 protein, one of the triple block proteins involved in local spread of white clover mosaic potexvirus (WClMV) strain O. The 13 kDa gene was mutated in a region of the gene that is conserved in all viruses known to process triple-gene-block proteins. The result indicated that transgenic *N. benthamiana* plants exhibited resistance to the O, M, and J strains of WClMV, including two other members of *Potexvirus* group; potato virus X, narcissus mosaic virus and the *Carlavirus* potato virus S whereas all plants from susceptible control lines became systemically infected (Beck *et al.*, 1994).

2.4 GENETIC TRANSFORMATION OF ORCHIDS

Many crop improvement programs are supplementing genome breeding methods with molecular breeding approaches (Tanaka *et al.*, 2005). This is because genetic

engineering, coupled with tissue culture techniques, has successfully introduced traits such as improved protein quality, novel flower color, and pest resistance in to various crops (Tanaka *et al.*, 2005; Goldbach *et al.*, 2003). For slow growing orchids, with their long generation times, limited available gene pool, and unknown genetics, it can be difficult to modify certain desirable traits such as flower color or disease resistance through sexual hybridization. Recently, only a few of transgenic orchid plants have been reported. Kuehnle and Sugii (1992) were the first to report the possibility of applying genetic transformation in *Dendrobium* orchid for potential trait improvement using particle bombardment. Subsequently, several orchid genera, *Phalaenopsis* (Anzai *et al.*, 1996; Yang *et al.*, 2003; Liao *et al.* 2004), *Cymbidium* (Yang *et al.*, 1999; Johnson *et al*, 1999), *Den. nobile* and *Den. phalaenopsis* (Men *et al.*, 2003b; Chan *et al.,* 2005), *Dendrobium* hybrids (Nan and Kuehnle, 1995; Tee and Maziah, 2005) and *Brassia*, *Cattleya* and *Doritaenopsis* (Knapp *et al.*, 2000), were transformed by bombardment using mainly GUS as the reporter system except Chia *et al.* (1994), where the firefly luciferase gene was inserted into *Dendrobium* orchid instead.

Transgenic orchid plants by using *Agrobacterium*-mediated transformation have also been studied in *Dendrobium* x Madame Thong-In (Yu *et al.*, 2001) and other *Dendrobium* hybrids, *Den. nobile* (Men *et al.*, 2003a), Oncidium (Liau *et al.*, 2003a,b), Phalaenopsis (Chan *et al.*, 2005; Belarmino and Mii, 2000).

Seed imbibition, electroinjection method and pollen tube-mediated transformation are also the alternative methods for transformation, but the low recovery rate of a putative transformant (0.005%) makes this method ineffective in *Dendrobium* orchids (Nan and

Kuehnle, 1995). The study in *Phalaenopsis* by using pollen tube-mediated transformation indicated that this method is a useful transformation method for producing transgenic *Phalaenopsis* plants (Hsieh and Huanng, 1995). In *Calanthe*, electrophoresis of protocorms resulted in a transformation frequency of about 50%, vs. a 3.3% by particle bombardment (Griesbach, 1994).

2.5 FLOWER COLOR MODIFICATION

Modification of flower colors through genetic engineering has been done in several crops such as *Petunia x hybrida* hort. Ex Vilm, *Zea mays* L. and *Antirrhinum majus* L. (Dooner *et al.,* 1991; Holton and Cornish, 1995; Mol *et al.*, 1998). While the biosynthetic pathways in these plants share a majority of common reactions, there are some important differences between the types of anthocyanins produced by each species. One major difference is that petunia does not normally produce pelargonidin pigments, whereas snapdragon and maize are incapable of producing delphinidin pigments (Holton and Cornish, 1995). The characterization of genetically defined mutations has enabled the sequence of enzymatic reactions in anthocyanin synthesis and their modification to be elucidated. Three different classes of anthocyanidins are responsible for the primary shade of the flower color; pelargonidin (orange to brick red), cyanidin (red to pink), and delphinidin (purple to blue). A key branch point of the anthocyanin biosynthetic pathway is centred around the intermediate DHK (dihydrokaempferol). The enzymes FLS (flavonol synthase), F3′H (flavonoid 3′-hydroxylase), F3′5′H (flavonoid 3′5′-hydroxylase) and DFR (dihydroflavonol 4- reductase) all utilize this substrate suggesting

that this region of the pathway is a key determinant of anthocyanin biosynthesis and thus flower color.

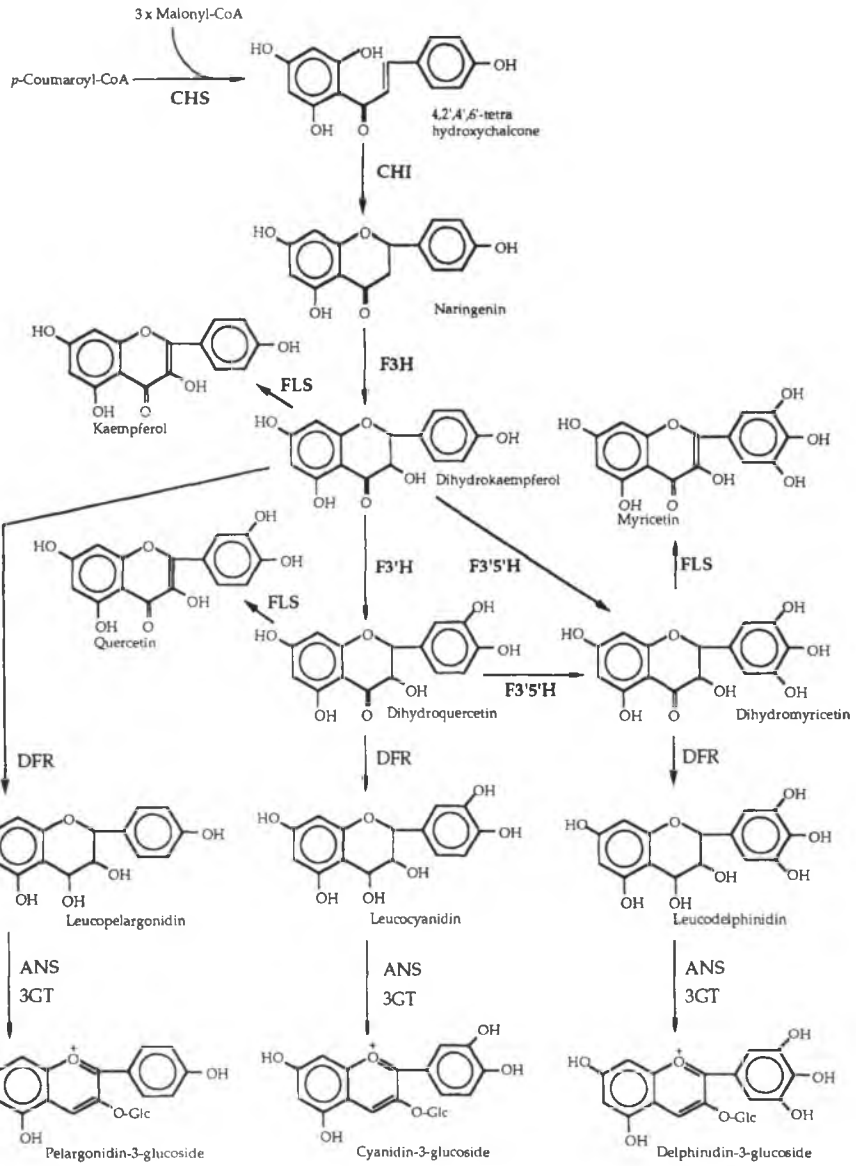

Figure 2.2 A diagrammatic representation of the flavonoid biosynthesis pathway (Holton and Cornish, 1995).

2.6 DIHYDROFLAVONOL- 4-REDUCTASE (DFR)

One of the biosynthetic enzymes, DFR catalyzes the NADPH - dependent conversion of three colorless dihydroflavonols - dihydrokaempferol (DHK), dihydroquercetin (DHQ) and dihydro- myricetin (DHM) to leucoanthocyanidins (Martin *et al.*, 1985; Reddy *et al.*, 1987; Holton and Cornish, 1995). These are subsequently converted to pelargonidin, cyanidin and delphinidin (Figure 2.2). The three substrates of DFR are very similar in their structure; the difference is only in the number of hydroxyl groups on the B phenyl ring, which is not the site of enzymatic action (Figure 2.2). Therefore, DFRs from many species can utilize all three substrates (Helariutta *et al.*, 1993; Stich *et al.*, 1992; Tanaka *et al.*, 1998; Johnson *et al.*, 2001). However, DFRs from some species such as *Petunia* and *Cymbidium* cannot reduce DHK efficiently, thus these species cannot produce pelargonidin-based orange flower color even if both F3′H and F3′5′H are absent (Forkmann and Ruhnau, 1987; Johnson *et al.*, 1999; Johnson *et al.*, 2001). In the first successful metabolic engineering study of the flavonoid pathway, the maize gene encoding dihydroflavonol 4-reductase (DFR) was introduced into petunia and gave a new orange phenotype (Meyer *et al.* 1987). This experiment initiated much work in this field. Mizutani *et al.* (2003) were able to engineer a red petunia line that normally accumulates cyanidin-based pigments to produce pelargonidin-based pigments (orange) by down regulation of the F3′H gene and expression of a rose DFR gene (Mizutani *et al.*, 2003).

Several of anthocyanin-specific enzymes involved in anthocyanin biosynthesis pathway have been identified by mutations. The *a1* gene from maize and the *pallida*

gene from *Antirrhinum majus* (snapdragon) encode dihydroflavonol 4-reductase (DFR) (Martin *et al.*, 1985; Reddy *et al.*, 1987). In *Petunia hybrida* five loci have been identified (*An1, An2, An6, An9*, and *An11*), which control the conversion of dihydroflavonols into anthocyanins in the flower limb (Doodeman *et al.*, 1984a; Gerats *et al.*, 1982). In *an6*⁻ mutants only DFR expression is impaired, whereas all other structural genes are normally expressed. Sine the *An6* locus maps to the same chromosome as one of the three *Dfr* genes it has been speculated that the *An6* locus encodes DFR (Beld *et al.*, 1989; Quattrocchio *et al.*, 1993). In contrast, mutations in *an1, an2, an4,* and *an11* affect the expression of *Dfr* and several other anthocyanin biosynthetic genes suggesting that they contain regulatory genes (Beld *et al.*, 1989; Gerats *et al.*, 1982; Kroon *et al.*, 1994; Quattrocchio *et al.,* 1993).

2.7 FLAVONOID 3′- HYDROXYLASE (F3′H)

The enzyme flavonoid 3′-hydroxylase (F3′H) in plants belongs to the cytochrome P450 superfamily, and its cDNA was first isolated from petunia (Brugliera *et al.*, 1999). The F3′H hydroxylates the 3′ position of the B-ring of naringenin and dihydrokaempferol to produce eriodictyol and dihydroquercetin, respectively, and the latter two products are precursors of cyanidin (Harborne, 1994; Holton and Cornish, 1995; Mol *et al.*, 1998; Shirley, 2001; Hoshino *et al.*, 2003) and also catalyzes the hydroxylation of naringenin and dihydrokaempferol in the 3′ position of petunia (Stotz *et al.*, 1985). The *Ht1* expresses in the limb and tube of the corolla, whereas *Ht2* expresses only in the corolla tube (Wiering, 1974). In maize, the F3′H gene was controlled by the *Pr* locus (Cone *et al.*, 1986). The aleurone of *Pr* plants is purple due to the accumulation of mostly

cyanidin glucoside, whereas the aleurone of *pr* plants is red due to accumulation of mostly pelargonidin glucoside. In snapdragon, the gene *eosina* (*eos*) control the B-ring hydroxylation of flavonoids in the 3′ position (Forkmann and Stotz, 1981).

2.8 FLAVONOID 3′, 5′- HYDROXYLASE (F3′5′H)

Flavonoid 3′, 5′- hydroxylase (F3′5′H), a member of the cytochrome P450 family, is the key enzyme in the synthesis of 3′, 5′- hydroxylated anthocyanins, which are usually a prerequisite for the expression of blue or purple flowers (Su and Hsu, 2003). An early objective was to introduce blue delphinidin pigments into top selling flowers such as rose, chrysanthemum and carnation, which have a wide array of colors, but lack the ability to generate blue or purple flowers because they cannot synthesize F3′5′H substituted anthocyanins. The F3′5′H gene has been transformed into several plant species, including carnation and rose since its isolation from petunia (Holton *et al.*, 1993a; Fukui *et al.*, 2003). F3′5′H genes from petunia and lisianthus have been shown to direct production of a blue hue in flowers of petunia and tobacco (Holton *et al.*, 1993a, Shimada *et al.*, 1999). Introduction of a F3′5′H gene, isolated from Canterbury bells (*Campanula medium*) resulted in flowers with a greater percentage of delphinidin (99 % delphinidin) than when the petunia or lisianthus F3′5′H genes were introduced (Okinaka *et al.*, 2003). This is presumably due to more efficient enzyme activity of the *Campanula* F3′5′H. Transformation of a pink *Lobelia erinus* with a lisianthus F3′5′H gene under the control of a CaMV35S promoter produced blue colored flowers. Furthermore, a recent study indicates that a flower-specific cytochrome b5 was required for enhancing activity of F3′5′H in petunia (de Vetten *et al.* 1999). Expression of a petunia F3′5′H in a

carnation line that accumulated cyanidin-based pigments resulted in very low levels of delphinidin production and no dramatic effect on flower color (Brugliera *et al.*, 2000b). It appears that the introduced petunia F3′5′H could not efficiently compete with the endogenous carnation F3′H and DFR enzymes. However, when a petunia cytochrome b5 gene along with the petunia F3′5′H gene were expressed in the same carnation line the result was a dramatic improvement in the level of delphinidin production and a shift in the flower color from a variegated pink and red to variegated mauve and purple (Tanaka *et al.*, 2005). Florigene Ltd. and Suntory Ltd. have successfully developed a range of transgenic violet carnations by introduction of a F3′5′H gene together with a petunia DFR gene into a DFR- deficient white carnation (Mol *et al.*, 1999; Tanaka *et al.*, 2005). The petals of the engineered carnations contain predominantly delphinidin that native carnations do not produce. The transgenic violet carnations named Florigene Moondust and Florigene Moonshadow have been marketed in Australia, Japan, North America and UK after being granted general release permission.

Su and Hsu (2003) successfully cloned a putative P450 gene from *Phalaenopsis*, and transiently transformed it into the petals of *Phalaenopsis*. The results clearly suggested that the putative F3′5′H gene is able to alter anthocyanin pigment synthesis in the cells of petals. It is likely that it will play an important role in flower color modification of the ever-growing popular *Phalaenopsis* in the near future.

The introduction of new colors and forms through genetic modification is likely to become an integral part of the breeding of new ornamental varieties. Although application of these techniques to floriculture has been slow to progress in comparison

with the major crop species, there are now transgenic carnations for sale in several countries, and many more floriculture products are in development. In commercial *Dendrobium* hybrids, color is determined by anthocyanins and carotenoids. Blue, red and orange colors are noticeably absent, yet are highly desired by the growers and consumers (Kuehnle *et al.*, 2003). Chemical and molecular analyses were conducted to understand the molecular basis of flower color in *Dendrobium*. We have a program aimed to understanding the basis of the color variation observed in *Dendrobium* orchids, so that strategies for modifying flower color can be developed.

Mudalige-Jayawickrama *et al.* (2005) produced the first complete cDNA clone encoding *Dendrobium Dfr*, isolated from floral buds of cyanidin-lavender *Dendrobium* UH503 and pelargonidin K1224. The nucleic acid sequence of the cDNA was determined and shown to be 87 % similar to DFR of *Cymbidium* orchid and 84 % similar to *Bromheadia* orchid (Mudalige-Jayawickrama *et al.* 2005). The expression of the gene as characterized by Northern blot analysis of developmentally staged *Dendrobium* flowers showed high activity throughout flower bud development but lower activity in fully open flowers; it is absent in leaves.

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CHAPTER 3

FUNCTIONAL CHARACTERIZATION OF *DENDROBIUM* DIHYDROFLAVONOL 4 - REDUCTASE (*DFR*) IN A *PETUNIA HYBRIDA* MODEL

3.1 INTRODUCTION

In Hawaii, *Dendrobium* has become the single most valuable commercial flower, given their combined use for cut flowers, leis, and blooming potted plants (NASS, 2005). Classical breeding techniques have given rise to many commercially successful hybrids with attractive flower colors and forms, long vase life, fragrance, seasonality and desirable spray length. However, some colors such as orange-red and blue are missing from the *Dendrobium* flower color spectrum (Kuehnle *et al.,* 1997). Flower color is an important feature that adds to the aesthetic appeal of *Dendrobium* orchids.

Flower color is influenced by a combination of factors, *i.e.,* the type of pigments present in the flower, the translocation of pigments from the site of production and the pH of the cell vacuoles (Mudalige-Jayawickrama *et al.*, 2005; Kuehnle *et al.,* 1997). Common plant pigments are the anthocyanins that produce the red, red-purple and blue colorations; the carotenoids that produce the yellows, oranges and orange-reds; and the chlorophylls that impart green coloration (Forkmann, 1991). It is essential to understand the existing floral pigments in a plant before new color variations can be developed.

Dendrobium orchid species and hybrids in commercial-trade have flower colors corresponding to pink, red, maroon, orange, bronze, and brown listed in the RHS Color Chart (Royal Horticultural Society, London). The above stated colors contain anthocyanins based on cyanidin, with peonidin occurring as a minor pigment. The colors of the blue genotypes correspond to the RHS color group for light violet to purple and contain anthocyanins based on cyanidin. Peach colored flowers may be classified as red or red - purple and included pelargonidin glycosides. Predominant copigments were flavonol glycosides that included kaempferol, quercetin, myricetin, and methylated derivatives (Kuehnle *et al.*, 1997).

The synthesis of anthocyanins occurs through the phenylpropanoid pathway, which starts with the stepwise condensation of three units of acetate from malonyl - coA together with 4 - coumaroyl CoA to yield tetrahydroxychalcone (Heller and Forkmann, 1988). This reaction is catalyzed by the enzyme chalcone synthase (CHS), which is very important for flower color manipulation since this is the first step in phenylpropanoid biosynthesis. There are several other important enzymes such as chalcone isomerase (CHI), flavanone 3 - hydroxylase (F3H), flavonoid 3' - hydroxylase (F3'H), flavonoid 3', 5'- hydroxylase (F3'5'H), anthocyanidin synthase (ANS), flavonoid 3 – O – glucosyl-transferase and dihydroflavonol 4 - reductase (DFR) (Figure 3.1). The reduction of dihydroflavonols such as dihydrokaempferol (DHK), dihydroquercetin (DHQ), and dihydromyricetin (DHM) to their corresponding leucoanthocyanidin is the first committed step in anthocyanin biosynthesis and is catalyzed by the enzyme, DFR (Figure3.1). The color of the anthocyanin produced corresponds to the dihydroflavonol

substrate that is reduced by DFR. The substrate - specificity of DFR explains the absence of certain colors from some ornamental plants. For example, the *Petunia hybrida* DFR cannot catalyze the conversion of DHK to orange pelargonidin (Meyer *et al.*, 1987). Only a few pelargonidin - accumulating flowers are found in *Dendrobium* (Kuehnle *et al.*, 1997). In *Cymbidium* orchids, the substrate specificity of *Cymbidium* DFR was investigated by transforming a mutant *Petunia* line accumulating DHK as the major flavonol (Johnson *et al.*, 1999). Thin Layer Chromatography (TLC) of transformed lines indicated that *Cymbidium* DFR cannot efficiently reduce DHK and preferred DHQ as a substrate, resulting in the production of pink cyanidin instead of orange pelargonidin. Flower color can be changed by manipulating plant pigment composition either via conventional breeding techniques or via biotechnology, *i.e.*, via genetic engineering. It usually takes breeders three to five years to assess and select desired flower characteristics by conventional breeding (Yu and Goh, 2001). The color outcomes of conventional hybridization work are, however, often uncontrollable and limited as they depend on the combinations of the original colors in the parental lines. Furthermore, the long sexual cycle of orchids, lasting approximately three to four years per generation, makes the traditional breeding process a very long one. Based on these limitations, the biotechnological approaches of directly manipulating orchid flower color at the molecular level appear very attractive and promising. The identification of the genes involved in color formation coupled with knowledge of genetic transformation procedures has enabled the development of transgenic plants with altered or enhanced flower pigments (Tanaka *et al.*, 2005). In this study, the full-length clone of *Dendrobium* dihydroflavonol - 4 - reductase (*DenDfr*) (Mudalige-Jayawickrama *et al.*, 2005) from Dendrobium x

Jaquelyne Thomas 'Uniwai Prince' or UH503 and Dendrobium x Icy Pink 'Sakura' or K1224 were used to transform to white petunia line W80 has white fowers and accumulates predominantly DHK due to mutations at *an6* (*DFRA*, Huits *et al.*, 1994), *ht1* and *ht2* (Flavonoid 3'-hydroxylase, F3'H), *hf1* and *hf2* (F3'5'H), *fl* (controls flavonol synthesis), and *rt* (UDP rhamnose: anthocyanidin-3-glucoside rhamnosyltransferase) to investigate the substrate specificity of *DenDfr*. However, both *Dfrs* from UH503 and K1224 are 100% identical (Mudalige - Jayawickrama *et al.*, 2005).

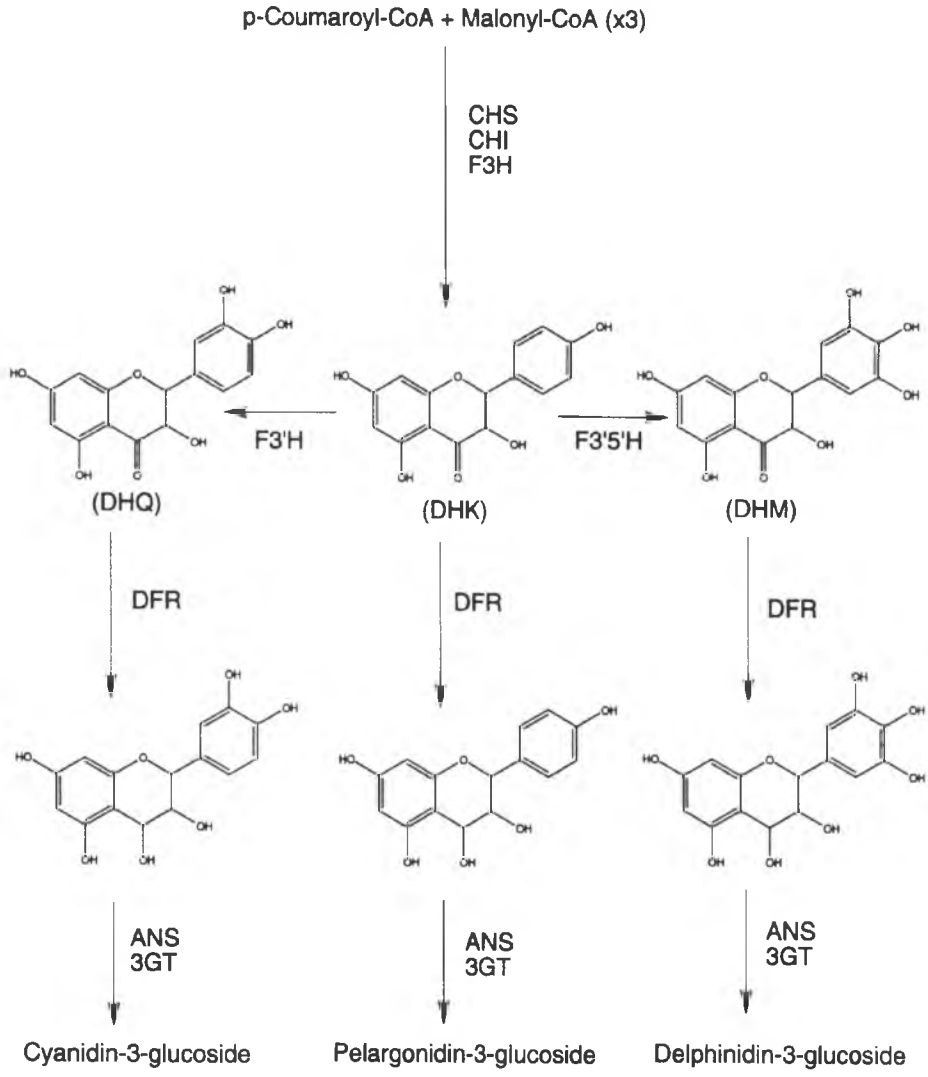

Figure 3.1 A schematic diagram showing the chemical reaction catalyzed by dihydro-flavonol 4 - reductase. Abbreviations used are; CHS: chalcone synthase, CHI: chalcone isomerase, F3H: flavanone 3-hydroxylase, F3'H: flavonoid 3'- hydroxylase, F3'5'H: flavonoid 3', 5'-hydroxylase, DFR: dihydroflavonol 4-reductase, ANS: anthocyanin synthase, 3GT: flavonoid 3-glucosyl-transferase (Johnson *et al.,* 2001).

3.2 OBJECTIVE

The objective of this study was to identify any substrate specificity of *DenDfr* by testing the hypothesis that *DenDfr* can reduce DHK (the less preferable substrate in *Cymbidium* orchid) producing pelargonidin - based orange flowers.

3.3 MATERIALS AND METHODS

3.3.1 Plant Material

3.3.1.1 *In vitro* and *ex vitro* germination

Petunia hybrida (W80) seeds were received courtesy of Dr. Ronald Koes (The University of Netherlands). To obtain plant material suitable for transformation, seedlings were germinated six to eight weeks prior to transformation on composted soil mix (Sun Gro Horticulture, Canada Ltd.) in 10-centimeter (4") plastic pots at ambient conditions in a greenhouse. *In vitro* cultures were also initiated by germinating sterilized W80 seeds *in vitro* on MS (Murashige and Skoog, 1962) medium, supplemented with 3% sucrose (w/v), and 0.7% granulated agar (w/v) (Difco^{TM}, Dickinson and Company) in nine centimeters Petri dishes (Fisher). Seeds were surface-sterilized by using 15% Clorox (w/v) (sodium hypochlorite 6%) plus 1% Polyoxyethylenesorbitanmonolaurate (Tween 20) (PhytoTechnology Lab., KS) for 5 min followed by three rinses in sterile distilled water. Seedlings were sub-cultured into magenta boxes (GA7), containing four seedlings per box and maintained on MS medium with a 16 h photoperiod of 19.0 $\pm$ 5 µmol m^{-2} sec^{-1} photon flux density provided by cool white and Gro - lux Sylvania fluorescent lamps (GTE Corp, Danvers, MA).

3.3.1.2 *Ex vitro* leaf surface sterilization

Ex vitro W80 leaf material was sterilized using Clorox (sodium hypochlorite 6% w/v) at concentrations of 5%, 10% or 15% and 1% Tween 20 for 5 min followed by three rinses in sterile distilled water. Sterilized leaves (10 leaves per plate) were plated adaxial surface up on modified MS media (full strength solidified MS supplemented with 2 mg L^{-1} 6-benzylaminopurine (BA) (Sigma) and 0.2 mg L^{-1} naphthalene acetic acid (NAA) (Sigma) for plant regeneration in nine centimeter sterile Petri-dishes (Fisher) under 16 h photoperiod of 19.0 $\pm$ 5 µmol $m^{-2}sec^{-1}$ photon flux density provided by cool white and Gro - lux Sylvania fluorescent lamps (GTE Corp, Danvers, MA). The data were observed every two weeks for two month by counting the contamination leaves (from either bacteria or fungal), dead leaves (brown color), and leaves producing shoots.

3.3.1.3 *In vitro* and *ex vitro* leaves materials

In vitro and *ex vitro* sources of leaf materials were compared to determine the best tissue source for use in regeneration and transformation experiments. Thirty young fully opened leaves were excised from *ex vitro* plants, surface sterilized and transferred to regeneration medium as described in section 3.5.1.1. In parallel, young, fully-opened leaves from *in vitro* plants were excised and transferred to regeneration medium under aseptic conditions.

3.3.1.4 Selection and regeneration of transformants

There is a variation in tolerance of antibiotics among different plants. This experiment determined the appropriate level of kanamycin for selecting petunia W80. Different levels of kanamycin (Agri - Bio) ranging from 50 – 200 mg L^{-1} were used for

selection of the transformants on regeneration media as described in section 3.3.1.2. The results were observed every week for one month by counting the number of pale bleaching leaves.

3.3.2 Gel Electrophoresis Methods

3.3.2.1 Agarose gel electrophoresis

Plasmid DNA digested with restriction endonucleases and PCR amplification products were size - fractionated by electrophoresis through 1% (w/v) agarose / 1 X TAE (40 mM Tris acetate, 1 mM EDTA, pH 8.0) and 0.002% Ethidium Bromide (Sigma). One - sixth volume loading dye containing 15 % (w/v) ficoll (Fisher), 0.25 % (w/v) bromophenol blue (Fisher), and 0.25 % (w/v) xylene cyanol FF (Sigma) in sterile distilled water was added to the samples prior to electrophoresis. DNA was visualized by UV illumination and photographed using a Polaroid (Foto Dynes) instant camera system.

3.3.2.2 Extraction of DNA from agarose gel

Both restricted and amplificated DNA fragments were excised using UV light for ethidium bromide from 1 X TAE agarose gels using a clean scalpel and DNA was extracted and purified using the Geneclean® II Kit (Q - BIOgene) exactly following the manufacturer's instructions.

3.3.3 Standard Cloning Methods

3.3.3.1 Ligation of DNA fragments

The PCR gel purified products were ligated into the pGEM® - T Easy Vector (Promega) following the manufacturer's instructions. The vectors are prepared with

appropriate restriction enzymes and by adding a 3' terminal thymidine to each end. These single 3'- T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into plasmids by preventing re-circularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases (Mezei and Storts, 1994; Robles and Doers, 1994). These polymerases often add a single deoxyadenosine, in a template-independent fashion, to the 3'- ends of the amplified fragments (Clark, 1988; Newton and Graham, 1994). The high copy number pGEM® - T Easy Vector contain T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide allows recombinant clones to be directly identified by color screening on indicator medium.

3.3.3.2 Dephosphorylation of DNA

To prevent self - annealing during ligation, 5' phosphate groups were removed from digested DNA using shrimp alkaline phosphatase (SAP) (Promega). The plasmid DNA was resuspended in 5 μL sterile distilled water, and 1.7 μL of 5X ligase buffer (NEB) was then added.

3.3.4 Extraction of Nucleic Acids

3.3.4.1 Plasmid DNA extraction

A single bacterial colony harboring plasmid DNA was inoculated in 2 mL of Luria - Bertani (LB) medium (1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.0% (w/v) sodium chloride, pH 7.0) containing the appropriate antibiotics, shaken at 250 rpm at 37°C for 16 h, and centrifuged at 13000 rpm for 30 sec. The supernatant was then removed and plasmid DNA extracted using a Quantum Prep® Plasmid Miniprep (Bio -

Rad) following the manufacturer's instructions. Plasmid DNA was quantified using a Spectronic 601 spectrophotometer (Milton Roy) and stored at -20°C.

3.3.4.2 RNA Extraction

Total RNA was extracted from 100 mg of leave tissue, using TRI REAGENT™ (Sigma) following the manufacturing manual, and stored at -80°C.

3.3.5 Polymerase Chain Reaction (PCR)

3.3.5.1 Adding enzyme recognition site using PCR

PCR was performed to identify and amplify genes and to add enzyme recognition sites using the primers 5'-GGTCTACCATGGAGAATGAGAAG-3' and 5'-GGTCGGATCCTCACTTAACAGC-3' to add *Nco*I and *Bam*HI sites, respectively. *DenDfr* was amplified from two different varieties of *Dendrobium* orchid; *Dendrobium* x Jaquelyn Thomas 'Uniwai Prince' (UH503) (*503Dfr*), and *Dendrobium* Icy Pink 'Sakura' (K1224) (*1224Dfr*). A concentration of 200 ng of genomic DNA was mixed into 50 µL of PCR reaction mix (1 x PCR buffer (Qiagen), 0.2 mM dNTPs, 1.0 mM magnesium chloride, 0.4 µM forward and reverse primers, 2 U HotStarTaq® DNA Polymerase (Qiagen). PCR cycling conditions were, Stage 1: 95°C for 15 min; Stage 2: 95°C for 1 min, 55°C for 1 min, 72°C for 1.30 min, for 25 cycles; Stage 3: 72°C for 7 min. Amplification products were visualized by electrophoresis on 1 x TAE agarose gel (section 3.3.2.1) and excised using a clean scalpel and extracted (section 3.3.2.2).

3.3.5.2 Reverse transcriptase PCR (RT-PCR)

Total RNA extracted from petunia tissues (section 3.3.4.2) was converted into complementary DNA (cDNA) using a reverse transcriptase kit (Invitrogen) following the manufacturer's instructions. The specific primers 5'-ATTTCTTGACGGTTTTTGCT TGC -3' and 5'- GGAAGTTCATTTCATTTGGAGAG -3' were used to amplify the *SnpDfr* gene cDNA, and the specific primers 5'-GGTCTACCATG GAGAATGAGAAG-3' and 5'-GGTCGGATCCTCACTTAACAGC-3' were used to amplify the *DenDfr* cDNA (both contained *503Dfr*s and *1224Dfr*s). PCR reactions and conditions as described (section 3.3.5.1).

3.3.5.3 Screening of bacterial colonies for plasmids using PCR

A single bacterial colony was picked from a transformation plate and mixed into 50 µL of PCR reaction mix (1X PCR buffer (Qiagen), 0.2 mM dNTPs, 1.0 mM magnesium chloride, 0.4 µM forward and reverse primers, and 2 U HotStarTaq® DNA Polymerase (Qiagen). PCR cycling conditions were, Stage 1: 95°C for 15 min; Stage 2: 95°C for 1 min, 55°C for 1 min, 72°C for 1.30 min, for 25 cycles; Stage 3: 72°C for 7 min. Amplification products were visualized by electrophoresis on 1% TAE agarose gels (section 3.3.2.1).

3.3.6 Genetic Transformation of Plants

3.3.6.1 Transformation of *Escherichia coli* (*E. coli*)

Competent cells of *E. coli* (50 µL, TOP10, Invitrogen) were thawed and transferred to a 1.5 mL Eppendorf tube containing 4 µL of ligation reaction (vector: insert

DNA ratios, 1: 3 and 1 x T4 DNA ligase buffer solution, Promega). This mixture was incubated for 30 min on ice, incubated at 42°C for 30 sec, returned to ice for 2 min and 250 µL of room temperature SOC medium (2.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) sodium chloride, 20 mM glucose, 2.5 mM potassium chloride, 10 mM magnesium chloride, pH 7.0) were added to each reaction and subsequently incubated at 37 °C at 250 rpm for one hour. Cells were plated out onto LB agar plates (1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.0% (w/v) sodium chloride, pH 7.0, Bactoagar 15 $g L^{-1}$) containing the appropriate antibiotic for the construct in use and cultured overnight at 37°C.

3.3.6.2 Nuclear transformation of *Petunia*

3.3.6.2.1 By *Agrobacterium*-mediated transformation

Leaves of *Petunia* W80 taken from *in vitro* cultures (section 3.3.1.1) were placed with the adaxial surface up on a plate containing MS regeneration medium (full-strength MS medium supplemented with 2 $mg L^{-1}$ NAA and 0.2 $mg L^{-1}$ BA) and kept under 16 h light at 25°C for 24 h prior to transformation.

Single colonies of *Agrobacterium tumefaciens* containing pLN13 (*SnpDfr*) (section 3.3.8.1), pBINPLUS with *503Dfr* (section 3.3.8.2) and pBINPLUS with *1224Dfr* plasmids (section 3.3.8.3) were inoculated into liquid YM medium (0.04 % (w/v) yeast extract, 1.0 % (w/v) mannitol, 1.7 mM sodium chloride, 0.8 mM magnesium sulfate 7 - hydrate, 2.2 mM potassium phosphate diabasic, trihydrate, pH 7.0), and grown to log-phase (OD_{600nm} of 0.8 – 1.0) at 28°C. The culture was then centriuged at 5500 rpm for 20 min and resuspended in 10 mL of YM medium aliquots. Prior to treatment, several

incisions were made on each leaves, follow by incubation with 10 ml *Agrobacterium* solution for 10 min. Leaves were then blotted on sterile paper towels to remove excess *Agrobacteriu,* placed on regeneration media, and maintained in darkness for two day at 25°C. Two days after co-culture with *Agrobacterium* the leaf explants were transferred to regeneration medium supplemented with 500 $mg.L^{-1}$ carbenicillin (Phytotechnology) and appropriate level of kanamycin (Agri - Bio) and sub-cultured on the same medium until shoots formed. Shoots were subsequently cultured on rooting medium (full-strength MS lacking growth regulators) supplemented with 250 $mg\,mL^{-1}$ carbenicillin and 50 $mg\,L^{-1}$ kanamycin. Transgene integration was confirmed in regenerated plantlets by RT-PCR (section 3.3.5.2).

3.3.6.2.2 By Microprojectile Bombardment using Particle Inflow Gun

The plasmid pUBQ3-GUS, containing the *Arabidopsis* ubiquitin3 (UBQ3) (courtesy of Sanford Scientific Inc., NY) promoter driven β - glucuronidase GUS (*gus*A) gene was used for bombardment of commercial petunia leaves and flowers and also *Dendrobium* X Jaquelyne Thomas 'Uniwai Supreme' (UH232) flowers. Plasmid DNA was extracted using plasmid DNA isolation miniprep kits (Qiagen, Valencia, CA). The plasmid containing the *gus*A reporter gene was coated onto 1.6 µM gold particles (Bio-Rad laboratories, Hercules, CA). Gold particles (50 mg, 1.6 µM in diameter, Bio-Rad) were sterilized by vortex in 500 µL of 95 % ethanol for 15 min. The gold particles were allowed to precipitate at room temperature, after which the ethanol was discarded and the gold particles were washed three times in 500 µL sterile distilled water. After the last wash, the gold particles were resuspended in 500 µL sterile distilled water. A 50 µL

aliquot of the suspension was removed and 5 µg of plasmid DNA, 20 µL of 0.1 M spermidine, and 50 µL of $CaCl_2$ were added, in that order, with vigorous pipetting between additions. The mixture was then vortexed at room temperature and allowed to precipitate on ice for 10 min., after which 85 µL of supernatant was removed. The gold was resuspended very well by vortex vigorously before use. For each bombardment, 5 µL of the particle suspension was pipetted onto a Swinnex filter holder (Millipore, Ireland). Microprojectile bombardments were carried out using Particle Inflow Gun (PIG) particle delivery system (Kiwi Scientific) at a vacuum pressure of 14 psi, helium pressure of 60 psi, pulse length at 30 msec, and target tissue placed on the 3rd stage from the bottom. All plant tissues were place on full strength MS medium for 24 hour before analysis using the *β* - glucuronidase (GUS) histochemical assay.

3.3.7 Analysis of Reporter Gene Activity

3.3.7.1 β - glucuronidase (GUS) histochemical assay

Leaves, roots and flowers from putative transgenics containing the *β* - glucuronidase GUS (*gus*A) gene were placed in X - Gluc solution (0.1% X-gluc (GOLD Bio *Technology*, Inc., MO), 20% methanol, and 80% GUS staining buffer (100 mM $NaPO_4$, pH 7.0, 1% TritonX, 1% DMSO, and 10 mM EDTA) and incubated at 37°C for 24 h (appendix). Chlorophyll was removed from the stained tissues by incubation in 70% ethanol. The blue precipitate synthesized as a result of GUS activity was observed by visualization of the blue spots.

3.3.8 Vector Construction

To investigate anthocyanidin production and substrate specificity in petunia, W80 flowers were transformed with three different dihydroflavonol 4 - reductase (DFR) genes: *Antirrhinum majus* (*SnpDfr*), *Dendrobium* x Jaquelyn Thomas 'Uniwai Prince' (UH503) (*503Dfr*), and *Dendrobium* x Icy Pink 'Sakura' (K1224) (*1224Dfr*).

The series of corresponding *Agrobacterium* transformation vectors were created using standard cloning methods as described (section 3.3.3).

3.3.8.1 Construction of three transcription units containing the *SnpDfr* , *503Dfr*, or *1224Dfr* gene

Plasmid pLN13 (13 kb) comprising a full - length snapdragon (*Antirrhinum majus*) *SnpDfr* cDNA (1.6 kb), which encodes a DFR that readily accepts dihydrokaempferol as a substrate (Holton and Cornish, 1995), cloned in the binary vector pGA643 (An *et al.*, 1985) was kindly supplied by Kevin Davies, (Crop and Food Research, Palmerston North, New Zealand). Plasmid pLN13 has Cauliflower Mosaic Virus (CaMV) 35S promoters driving both the *SnpDfr* and *nptII* (neomycin phosphotransferase) genes and also has pTi 5'7' terminator.

The *1224Dfr* was amplified with specific primer (section 3.3.5.1) to add *Nco*I at the 5' and and *Bam*HI at 3' termini. The PCR product were cut with *Nco*I and *Bam*HI and sub-cloned into the plasmid vector pBI525 (Courtesy of Dr. William L. Crosby, Plant Biotechnology Institute, Canada) in the sense direction under the control of the double Cauliflower Mosaic Virus (CaMV) 35S promoter (35S-35S) (Figure 3.2) The promoter-gene-terminator regions were removed from pBI525 by partial digestion with *Hind*III and

*Eco*RI and further sub-cloned into *Agrobacterium* vector pBINPLUS (pBIN$^+$) (van Engelen *et al.*, 1995).

The *503Dfr* was amplified with specific primer (section 3.3.5.1) to add *Nco*I at the 5' and and *Bam*HI at 3' termini. The PCR product was cut with *Nco*I and *Bam*HI and sub-cloned into the plasmid vector pBI525 in the sense direction under the control of the ubiquitin3 (UBQ3) promoter from *Arabidopsis thaliana* (Figure 3.3) (substituting promoter by Dr. Michele Champagne in 2002 by replacing the HindIII-NcoI fragment of pBI525 containing the double 35S promoter with a HindIII-NcoI fragment from pUBQ3NcoIGUS (Courtesy of Dr. Judy Callis via Sanford Scientific, Inc.) containing the Ubiquitin 3 promoter). The promoter-gene-terminator regions were removed from pBI525 by partial digestion with *Hind*III and *Eco*RI and further sub-cloned into *Agrobacterium* vector pBINPLUS (pBIN$^+$) (van Engelen *et al.*, 1995).

Subsequently, all three plasmids were transformed to *Agrobacterium tumefaciens* strain LBA4404 (Life Technologies, Inc., MO) by electroporation using a BTX Electro Cell Manipulator®600 following manufacturer protocol (BTX Inc., CA)

3.3.9 Anthocyanin Analysis

Petals of *petunia* W80, K1224, and UH503 were hydrolyzed in 3N HCl at 100°C in a heat block for 45 min before anthocyanins were extracted according to the method of Kuehnle *et al.* (1997). Extracted samples were analyzed by thin layer chromatography (TLC) using cellulose MN300 plates (Analtech, Newark, DE) in TBA (*t*-butanol/acetic acid/water, 3:1:1) as described in Kuehnle *et al.* (1997) and Formic solvent (HCl/formic acid/H_2O, 3:30:10) as described in Irani and Grotewold (2005). Standards for TLC

analysis (cyanidin chloride, delphinidin chloride, malvidin chloride, pelargonidin chloride, and peonidin chloride) were obtained from Apin Chemicals (Oxon, UK).

Plasmid construction of a transcription unit containing the *1224Dfr* gene

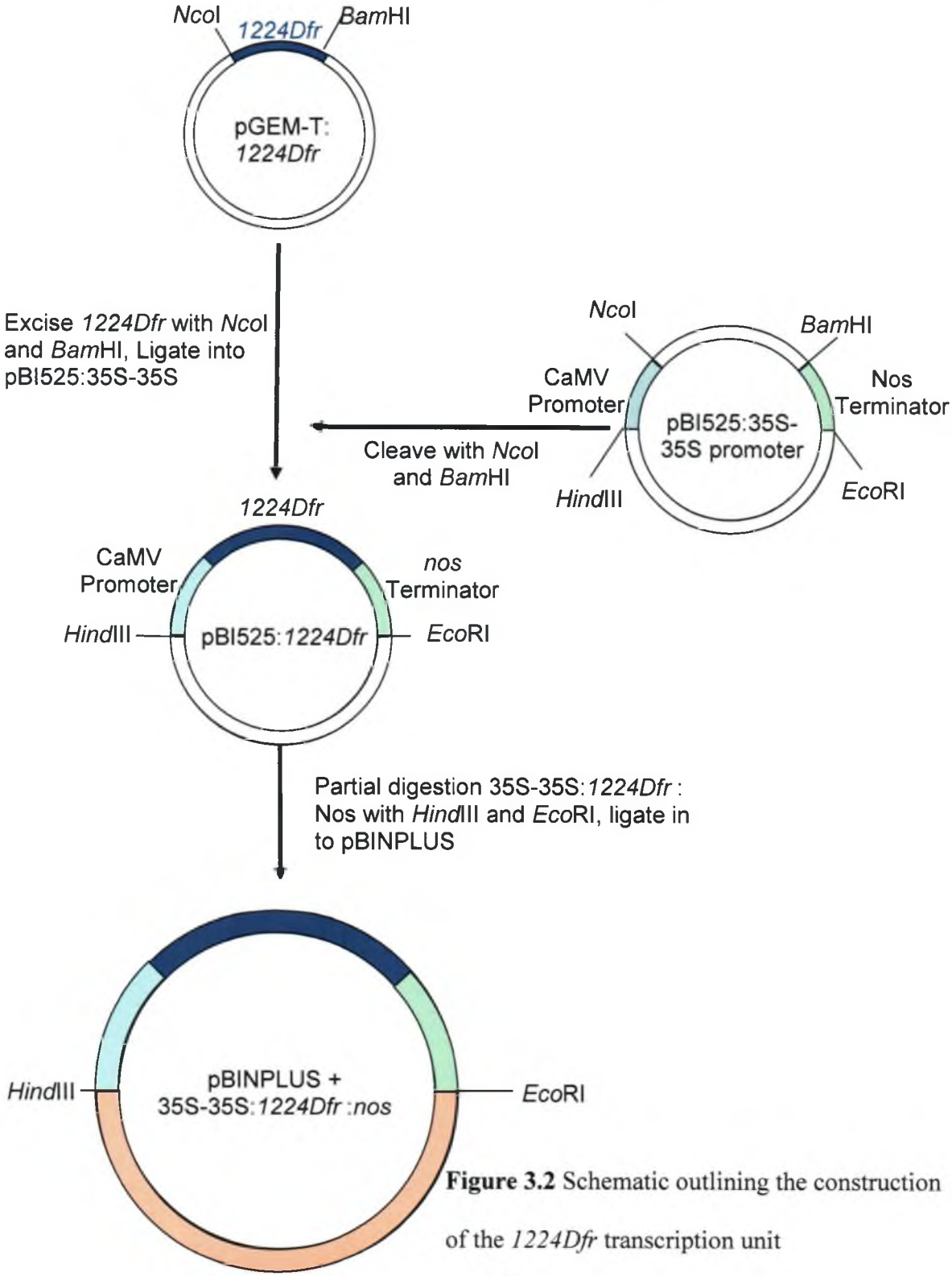

Figure 3.2 Schematic outlining the construction of the *1224Dfr* transcription unit

Plasmid construction of a transcription unit containing the *503Dfr* gene

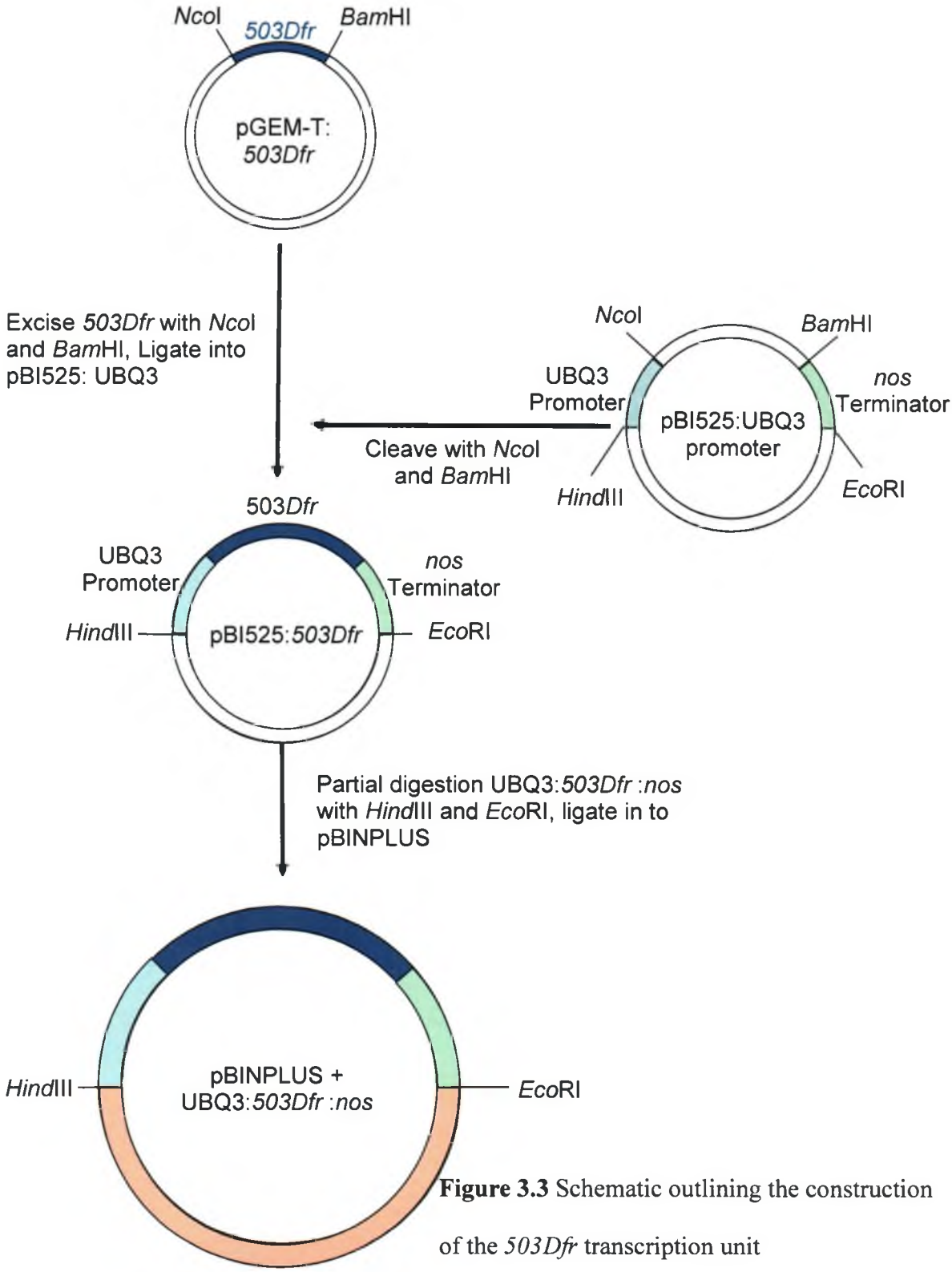

Figure 3.3 Schematic outlining the construction of the *503Dfr* transcription unit

3.4 RESULTS

3.4.1 *Petunia* seed germination

We compared *in vitro* and *ex vitro* seed germination rates. Results after 6 weeks indicated that germination *in vitro* gave a much higher number of seedlings compared to germination *ex vitro* in potting mix (Table 3.1).

Table 3.1 Comparison of germination rates between *In Vitro* and *Ex Vitro* condition.

Germination	No. of Seeds	% Germination
In Vitro on MS medium	50	90
Ex Vitro on potting mix	50	40

3.4.2 Optimization of *ex vitro* leaf surface sterilization

The leaves from *ex vitro* plants have to be surface-sterilized before use. This experiment used three different concentration of Clorox (section 3.3.1.2). Results indicated that surface sterilization with 10% clorox generated a high number of leaves forming shoots and a low level of bacterial and fungal contamination (Table 3.2). All leaves that survived in each experiment were able to produce shoots in regeneration medium.

Table 3.2 Optimization of leaf surface sterilization using three different concentrations of Clorox.

(%) Clorox	No. of	% contaminated	% bleaching	% shoots
5	30	56.7	0	43.3
10	30	16.7	13.3	70.0
15	30	0	63.3	36.7

3.4.3 *In vitro* and *ex vitro* leaves materials

We used two different sources of leaves material and compared between the same amount of starting leaf material from *in vitro* and *ex vitro* (sterilized using 10% Clorox, 5 min). Results indicated that *in vitro* leaf materials gave higher recovery rate of shoots on selection medium supplement with 100 mg L^{-1} kanamycin and no contamination after co-cultivation with *Agrobacterium*. So we preferred to use the leaf from *in vitro* rather than *ex vitro* (Table 3.3).

Table 3.3 Comparison of two different sources of leaf materials for shoot recovery after *Agrobacterium* transformation

Source	No. of leaves	% of contaminated leaves	% of dead leaves	% leaves producing shoots
Ex Vitro	30	13.3	13.3	73.3
In Vitro	30	0	0	100.0

3.4.4 Selection of transformants

Antibiotic selection is a crucial step in plant transformation methods. There was variation in mortality under different selection regimes as shown in Table 3.4, with no survival on 200 mg L^{-1}. The survival rate is determined by counting the leaves with at least some green patches remaining after culture on shoot regeneration medium with different levels of kanamycin.

Table 3.4 Sensitivity of wild type *Petunia hybrida* after one month culture on shoot regeneration medium with different levels of kanamycin.

Kanamycin (mg L^{-1})	No. of leaves	% Kill
0	30	0
50	30	60
100	30	75
150	30	97.5
200	30	100

3.4.5 Transformation and Regeneration

Based on the result from the previous experiment, *in vitro* petunia leaves were used for *Agrobacterium* transformation. Different levels of kanamycin ranging from 50 – 200 mg L^{-1} were used for selection of transgenic shoots. The result from the killed curve showed that regeneration media with 100 mg L^{-1} kanamycin killed 75% of petunia leaves, whereas 150 mg L^{-1} kanamycin killed 97.5% of petunia leaves. A concentration of 100

$mg\,L^{-1}$ kanamycin was chosen for selecting transformed plants because this concentration killed most of the non-transformed plants, but was not toxic enough to kill the transformants. A 25% survival rate was obtained from this selection protocol (Table 3.4).

Plantlets were regenerated from co-cultivated leaves on full-strength solidified MS media, supplemented with 3% sucrose (W/V), 0.7% granulated agar (W/V), 2 $mg\,L^{-1}$ BA, and 0.2 $mg\,L^{-1}$ NAA, while a kanamycin level of 100 $mg\,L^{-1}$ was used as an antibiotic selectable marker and carbenicillin level of 500 $mg\,L^{-1}$ was used according to Firoozabady and Kuehnle (1995) for avoiding *Agrobacterium* re-growth. Plates were sub-cultured every 2 – 3 weeks, because the antibiotics gradually become ineffective.

After the first shoots developed to 1–1.5 cm length, the shoots from the explant/callus were cut and placed upright in rooting medium in Magenta boxes. The rooting medium was full-strength solidified MS media lacking growth regulaters, supplemented with 3 % sucrose (W/V), 0.7 % granulated agar (W/V), 50 $mg.L^{-1}$ kanamycin, and 250 $mg.L^{-1}$ carbenicillin. After 1–2 weeks, the transformed plants produced roots. These were maintained *in vitro* without subculturing until they flowered (Figure 3.4). Different transformations produced different colors ranging from white, light pink, pink, salmon to orange as shown in Figure 3.5A.

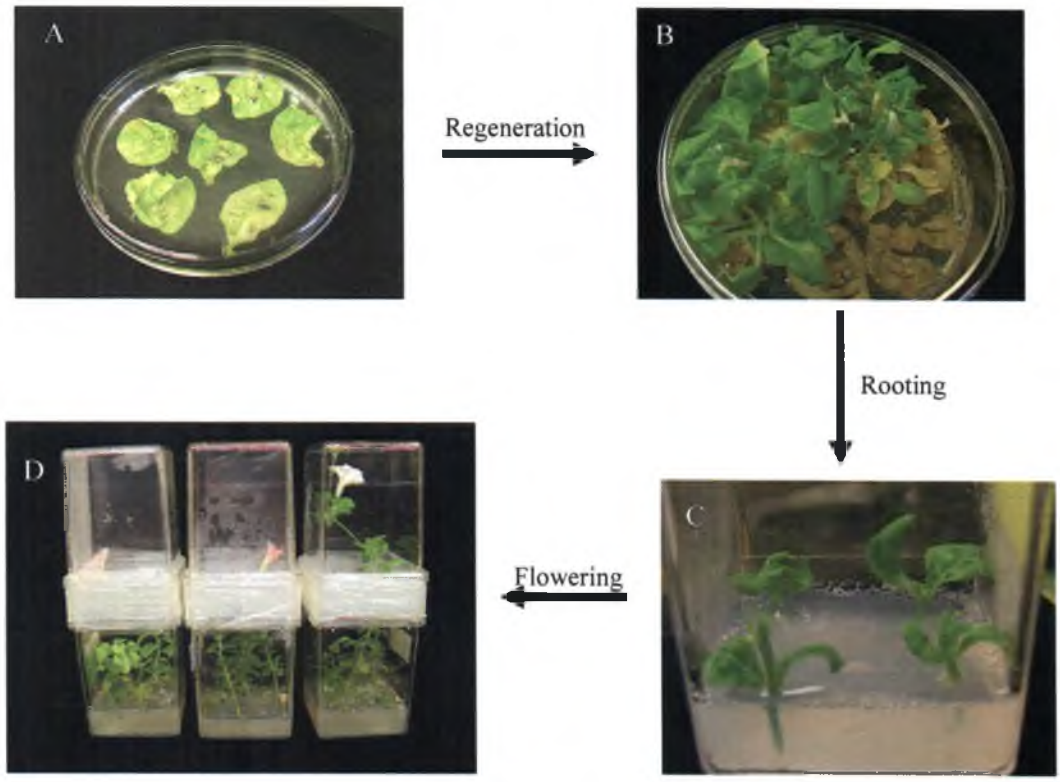

Figure 3.4 Schematic showing key stages in nuclear transformation. Introduction of nuclear transformation vector DNA into donor leaf material via *Agrobacterium* transformation. (A) Petunia leaves after co-cultivation for 10 min. with *Agrobacterium*. (B) Regenerating shoots on selection 8 – 10 weeks post co - cultivation. (C) Shoots are excised and transferred onto rooting medium. (D) Flowering *in vitro* of putative nuclear transformed lines cultured in the presence of 100 mg L^{-1} kanamycin.

A

B

Gene Construct	Plants Flowering light pink- orange	Plants Flowering white	Total (plants)
pLN13:35S:*SnpDfr*:pTi 5'7'	10	4	14
pBin^{+}:UBQ3:*503Dfr*:*nos*	0	21	21
pBin^{+}:35S-35S:*1224Dfr*:*nos*	9	9	18

Figure 3.5 A: Examples of transgenic plants of *Petunia* W80 flowers ranging from light pink to dark orange. W80 normally is white.

B: Flower colors of individual *Petunia* W80 plants after transformation with different DFR constructs.

3.4.6 RT-PCR Analysis

Specific primers were designed to complement the *DenDfr* and *SnpDfr* sequences and used in RT-PCR (section 3.3.5.2). Total RNA isolated from petunia leaves and flowers from plants transformed with 35S:*SnpDfr* , UBQ3: *503Dfr* and 35S-35S:*1224Dfr* were used as template in the RT-PCR reaction (Table 3.5). We have used 12, 14 and 21 individual plants to test the expression of the transgene 35S-35S:*1224Dfr*, 35S:*SnpDfr* and UBQ3:*503Dfr*, respectively. The RT-PCR results indicated that all petunia W80s transformed with 35S-35S:*1224Dfr* and 35S:*SnpDfr* were positive for the expression of the inserted genes in the leaf tissue, including plants with white flowered phenotype (see Table 3.5). There was no flower color change in any petunia W80 plants transformed with UBQ3:*503Dfr* gene (Table 3.5). Further analysis was performed by isolating RNA from white petunia W80 flowers transformed with 35S-35S:*1224Dfr*, 35S:*SnpDfr,* and UBQ3:*503Dfr* genes. The result showed that three individual white flowers transformed with 35S-35S:*1224Dfr* and four individual white flowers transformed with 35S:*SnpDfr* were positive for the expression of *Dfr*, whereas there was an undetectable level of expression in all 21 white flowered plants resulting from treatment with UBQ3:*503Dfr* genes. To test whether this result was due to the promoter used (UBQ3), we used a β-glucronidase (X-Gluc) histochemical assay to assess expression of the *gusA* gene driven by the UBQ3 promoter. We used both *Agrobacterium*-mediated gene transfer and particle inflow gun to deliver the scorable marker (*gusA*) gene. A β-glucronidase (X-gluc) histochemical assay was carried out as described in section 3.3.7.1 to assess the expression of the *gusA* gene in flower and leaf tissue. Blue staining was observed in both

leaves and flowers of petunia W80 transiently transformed with UBQ3/*gusA* by particle inflow gun, whereas there was an undetectable level of blue staining in flower tissue of petunia W80 transformed with UBQ3/*gusA* by *Agrobacterium*-mediated gene transfer (Figure 3.6)

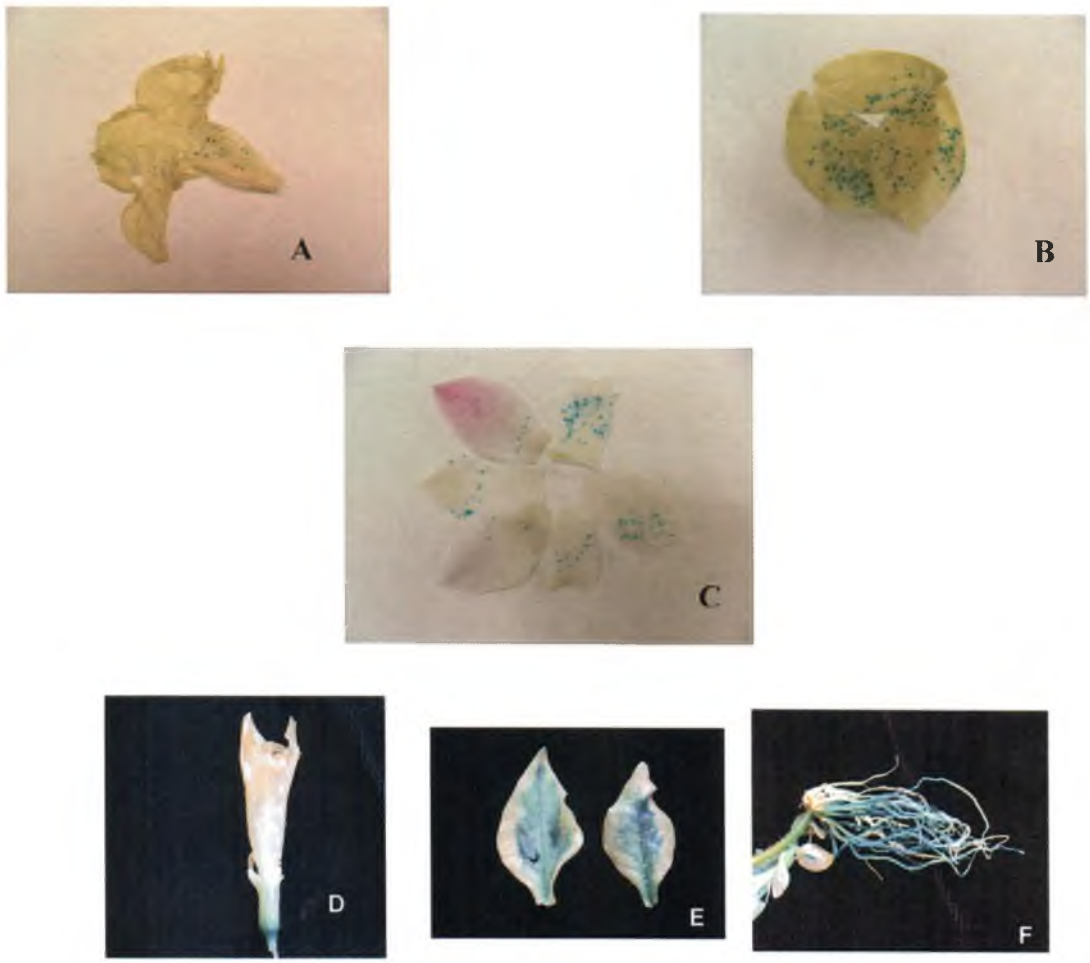

Figure 3.6 Histochemical analysis of *gusA* expression in transgenic petunia W80 line with UBQ3-*gusA* using transient and stable gene expression. (A), (B), and (C) are transient expression using particle inflow gun, whereas (D), (E), and (F) are tissues resulting from using *Agrobacterium*-mediated transformation. Light blue staining was observed on (A) Flower, whereas intense blue staining was observed in (B) petunia leaves and (C) *Dendrobium* flowers, (E) petunia leaves, and (E) petunia roots. No blue staining was observed on (D) petunia petals except on the base of the corolla (nectary) in this stable transformation.

Table 3.5 RT-PCR analysis of leaf and flower tissue of transgenic petunia transformed by *Agrobacterium tumefaciens* with *1224Dfr*, *SnpDfr* and *503Dfr* .

Construct	No. of Plants	Flower Color	Leaf RT-PCR	Flower RT-PCR
Db35S: 1224*Dfr*	1	Orange	+	ND
	2	Salmon	+	ND
	3	Pink	+	ND
	3	Light pink	+	ND
	9	White	+	+*
35S: *Snp-Dfr*	3	Orange	+	ND
	4	salmon	+	ND
	3	Light pink	+	ND
	4	White	+	+
Ubq3:503*Dfr*	21	White	+	-

* = 3 were RT-PCR positive; an additional 6 plants were not analyzed but rooted in Kanamycin 100 mgl^{-1}, suggesting they are transformant.

ND = not determined

3.4.7 Anthocyanin Analysis

The light pink to orange flowers of petunia W80 transformed with pLN13 (*SnpDfr*) and pBINPLUS (*1224Dfr*) were analyzed for identification of anthocyanidins. Newly-opened pigmented flowers from each transformation were collected and were then extracted in acid and analyzed by thin layer chromatography (TLC) against known standards (Figure 3.7). TLC analysis demonstrated that those flowers with the *snpDfr* contained mainly pelargonidin pigment, whereas those flowers with orchid *Dfr* contained a mixture of pelargonidin, cyanidin, and delphinidin according to retention factor (R_f) as shown in Table 3.6. Pelargonidin is derived from dihydrokaempferol (DHK), cyanidin is derived from dihydroquercetin (DHQ) whereas, delphinidin is derived from dihydromyricetin (DHM) precursor.

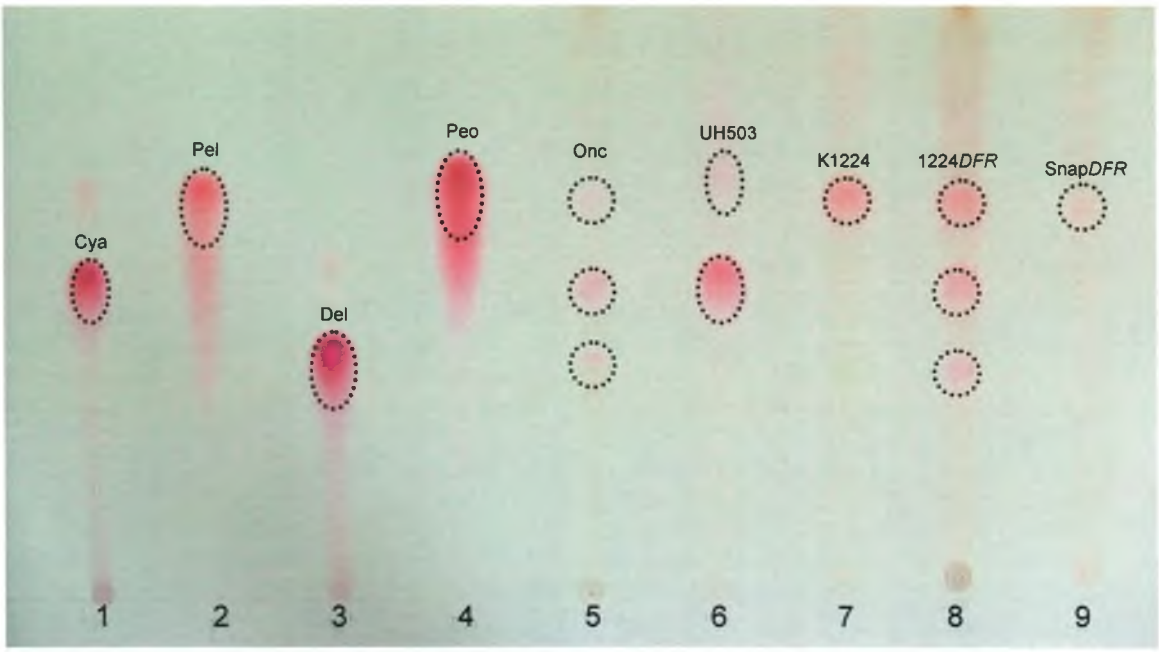

Figure 3.7 TLC analysis of flower pigments in petunia for orchid and snapdragon DFRs in TBA solvent. 1 = Cya: Cyanidin Standard, 2 = Pel: pelargonidin Standard, 3 = Del: Delphinidin Standard, 4 = Peo: Peonidin Standard, 5 = Extract of 3 corollas from the W80 transformed with Onc*Dfr* , 6 = Extract of 5 petals from UH503, 7 = extract of 10 petals from K1224, 8 = Extract of 6 corollas from W80 transformed with 1224*Dfr*, 9 = Extraction of 4 corollas from the W80 transformed with Snap*Dfr*.

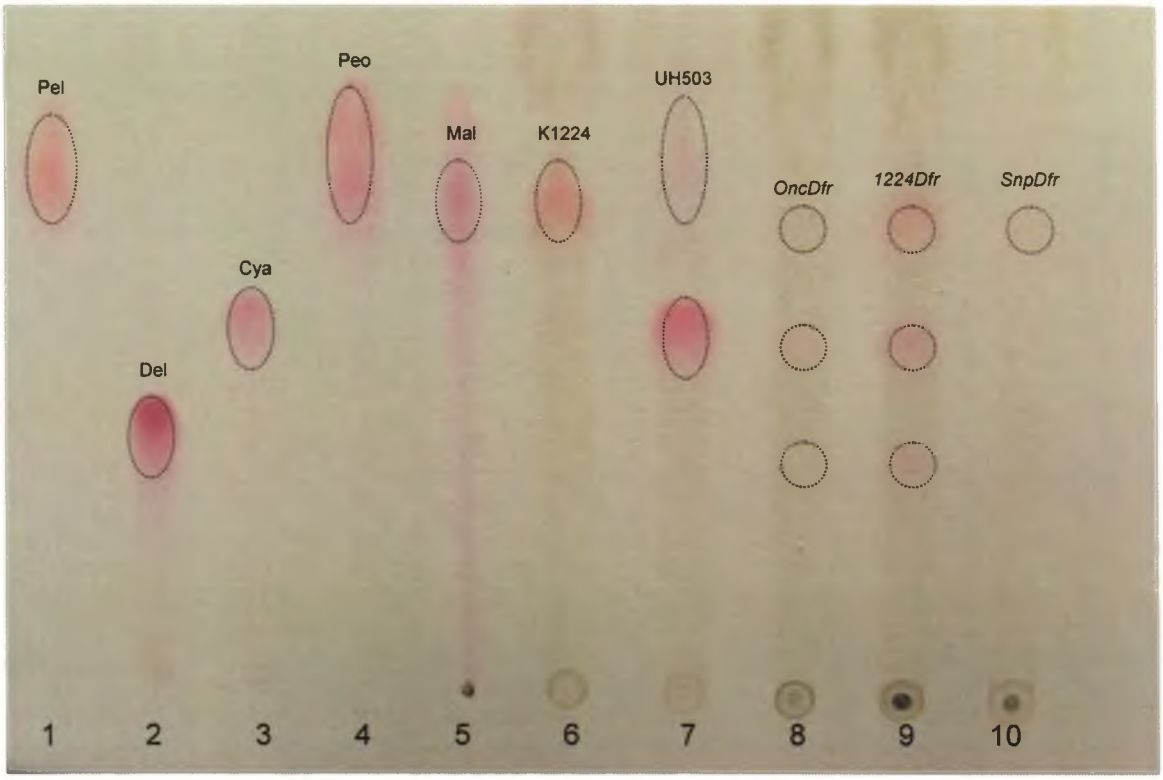

Figure 3.8 TLC analysis of flower pigments in petunia for orchid and snapdragon DFRs in Formic Acid solvent. 1= Pel: pelargonidin Standard, 2 = Del: Delphinidin Standard, 3 = Cya: Cyanidin Standard, 4 = Peo: Peonidin Standard, 5 = Malvidin Standard. 6 = extract of 10 petals from K1224, 7 = Extract of 5 petals from UH503, 8 = Extract of 3 corollas from the W80 transformed with Onc*Dfr*, 9 = Extract of 6 corollas from W80 transformed with 1224*Dfr*, 10 = Extraction of 4 corollas from the W80 transformed with Snap*Dfr*.

Table 3.6 R_f values of the anthocyanidins analyzed by TLC run in TBA solvent.

Anthocyanidins	R_f	UH503	K1224	1224*Dfr*	Snap*Dfr*	Onc*Dfr*
Cyanidin	0.50	0.50		0.50		0.50
Pelargonidin	0.63		0.63	0.63	0.63	0.63
Delphinidin	0.37			0.36		0.37
Peonidin	0.66	0.65				

Table 3.7 R_f values of the anthocyanidins analyzed by TLC run in Formic Acid solvent.

Anthocyanidins	R_f	UH503	K1224	1224*Dfr*	Snap*Dfr*	Onc*Dfr*
Cyanidin	0.45	0.45		0.44		0.44
Pelargonidin	0.60		0.60	0.61	0.60	0.61
Delphinidin	0.33			0.32		0.32
Peonidin	0.62	0.61				

3.5 DISCUSSION

3.5.1 Transformation and Regeneration

From our experiments, results indicated that *in vitro* leaves are more convenient to use and also had improved transformation efficiency over *ex vitro* leaves. Surface sterilization of the *ex vitro* leaves prior *Agrobacterium* transformation caused damage to the tissue, leading to less recovery regeneration rate causing the low transformation efficiency compared to the leaves from *in vitro* culture. These results are consistent with *Agrobacterium*-mediated transformation of soybean (*Glycine max* (L.) Merr.), in which the use of high vigor explants and minimum surface sterilization duration improved transformation efficiency (Paz *et al.*, 2004).

Selection is another crucial step in plant transformation since this is the key to separate the transformants from the non-transformed plants. Our experiment suggests that a 15 – 20 % survival rate on wild type control leaves will allow a good selection pressure to kill most of the non-transformed plants, without being toxic enough to kill the transformants. However, this selection level does not eliminate all non-transformed plants. High levels of antibiotic for short periods of time might be too toxic to the plants, inhibiting regeneration even in transformed tissues. Our results indicted that a kanamycin concentration of 100 $mg.L^{-1}$on regeneration media and rooting media might be the best selection regime for petunia W80 leaves transformed with *Agrobacterium* LBA4404.

3.5.2 Color Genes and Plasmid DNA

In this experiment, first the *503Dfr* gene was cloned into PBI525, which has *Arabidopsis* UBQ3 promoter, and then sub - cloned into pBINPLUS. The *1224Dfr* gene was cloned into PBI525, which has double Cauliflower Mosaic Virus (CaMV db35S) promoter, and then sub-cloned into pBINPLUS. The plasmid pLN13: *SnapDfr* was used as a plasmid control. After that each plasmid was transformed to *Agrobacterium tumefaciens* strain LBA4404 by electroporation technique. The RT-PCR results revealed that *Petunia* W80 transformed with 35S-35S: *1224Dfr*: *nos* had the same color range from white to light pink to orange to salmon as W80 transformed with 35S: *SnapDfr*: pTi5'7'. The flower color observation indicated the transgene was being expressed even in white flower tissues. The reasons why the enzyme was not active in the petal tissue might be due to the enzyme not being made, or being made, but not being function. The RT-PCR analysis in UBQ3: *503Dfr*: *nos* construct did not show the expression in white flower tissues, but showed in the leaf tissues. Based on the DFR nucleotide sequence comparison between *Den.* x Icy Pink 'Sakura' (*1224Dfr* gene) and *Den.* x Jaquelyn Thomas 'Uniwai Prince' (*503Dfr* gene), there is one nucleotide different from each other at position 137 from C to T, according to sequence analysis at the University of Hawaii Biotechnology Core Facility, resulting in a change of one amino acid from thymidine to isoleucine. This might be one reason that *Petunia* W80 transformed with *503Dfr* gene did not show any color changing in its flowers whereas the one that transformed with *1224Dfr* gene led to the color changing from white to light pink - dark orange. Other than one nucleotide difference between the two sequences, the promoter region is also

different between these two constructs. The *503Dfr* gene was driven by *Arabidopsis* ubiquitin3 (UBQ3) promoter, whereas the *1224Dfr* gene was driven by double Cauliflower Mosaic Virus (CaMV) 35S promoter (35S-35S). The result from *Agrobacterium*-mediated transformation showed that there was GUS expression all over the plant except in the flower petals (Figure 3.6). The result from particle bombardment showed that light blue staining was observed in the petunia petals, whereas intense blue staining was observed in petunia leaves and orchid flowers. From this result, we can conclude that the UBQ3 promoter has different level of expression in different plant species and different plant tissues. This result was supported by the maize ubiquitin (ubi-1) promoter driven the marker gene *uidA*, coding for *β*-glucuronidase (GUS) in individual transgenic wheat (*Triticum aestivum* L.) lines from different wheat varieties showed the different level of expression in some of the lines. The expression was strong and constitutive in all tissue in some of the lines whereas, there were also transgenic lines in which GUS activity was restricted to only a few tissues (Rooke *et al.*, 2000). Variation in transgene expression levels between different species and promoters also depend on the different abundance of transcription factors, recognition of promoter sequences or intron splicing sites (Wilmik *et al.*, 1995; Yang *et al.*, 2003).

Sequence analysis of the UBQ3 promoter that we used in our experiment compared to *Arabidopsis thaliana* cDNA (Callis *et al.*, 1995) revealed an absence of an intron in the 5'- untranslated region. Introns are studied in many cases and the results indicated they have a large positive effect on gene expression in many organisms including nematodes, insects, and mammals (Buchmann and Berg, 1988; Chung and

Perry, 1989; Meredith and Storti, 1993; Okkema *et al.*, 1993). In plants, the inclusion of one or more introns in a gene construct usually leads to increased accumulation of mRNA and protein (Rose and Beliakoff, 2000). This was supported by Norris and Callis (1993), who isolated and determined DNA sequence for the 5'-flanking regions of three different *Arabidopsis thaliana* polyubiquitin genes. Results indicated that the expression of chimeric genes using the coding regions for the marker enzymes β-glucuronidase and firefly luciferase was 2.5 to 3 fold lower when the introns were removed (Norris and Callis, 1993).

3.5.3 Anthocyanin Analysis

The petunia W80 bears white flowers and accumulates DHK due to mutations at an6 (DFRA, Huits *et al.*, 1994), *ht1* and *ht2* (flavonoid 3' - hydroxylase, F3' H) *hf1* and *hf2* (F3'5'H), *fl* (controls flavonol synthesis), and *rt* (UDP rhamnose:anthocyanidin-3 - glucoside rhamnosyltransferase). When W80 was transformed with a *SnpDfr*, which can efficiently reduce the substrate DHK and DHQ (Holton and Cornish, 1995), light pink to orange flowers were produced (Figure 3.5), as reported in a previous experiment (Holton and Cornish, 1995). Similarly 18 independent W80 transformants containing the *1224Dfr* displayed the same range of color from white to light pink to orange. Based on TLC analysis, we identified the pigments in orchid *Dfr* plants as pelargonidin, cyanidin, and delphinidin. Our analysis confirms that *1224Dfr* can convert DHK efficiently to produce orange - colored pelargonidin. The *1224Dfr* was cloned from *Den.* x Icy Pink 'Sakura', which has pale orange flowers, which have been analyzed and identified as 90% pelargonidin and 10% peonidin (Kuehnle *et al.*, 1997). Conversely, *503Dfr* gene

from *Den.* x Jaquelyn Thomas 'Uniwai Prince' (UH503), which has lavender colored flowers, that were analyzed and identified as cyanidin (Kuehnle *et al.*, 1997). However, the sequence comparison between *Den.* x Icy Pink 'Sakura' (K1224) and *Den.* x Jaquelyn Thomas 'Uniwai Prince' (UH503) are 100% identical to each other (Mudalige - Jayawickrama *et al.*, 2005). They also shared an 83% nucleotide sequence identity with the *CymbidiumDfr* gene, whose corresponding enzyme does not efficiently reduce DHK to orange pelargonidin (Johnson *et al.*, 1999). A putative region that determines the substrate specificity of DFR based on sequence alignment of petunia, maize and snapdragon, was proposed by Beld *et al.* (1989). Johnson *et al.* (2001) further identified four unique amino acid residues from this region that determine the substrate specificity of *Petunia Dfr*. However, these four amino acids are not shared by *Cymbidium* and *Petunia*, despite their similarity in substrate specificity. This ruled out the possibility of these residues as the region of substrate specificity for the *Cymbidium Dfr*. Therefore, our results agree with the hypothesis that the type of anthocyanins produced by orchid *Dfr* may be determined by another region of the enzyme (Johnson *et al.*, 2001), different from that described in *Petunia*. Our results from the study of substrate specificity for *Dendrobium Dfr* suggested that an orange-pelargonidin color in the flower of *Den.* x Icy Pink 'Sakura' could be due to a mutation of the flavonoid 3'-hydroxylase (F3'H) enzyme could reduce the amount of available dihydroquercetin (DHQ), making dihydro-kaempferol (DHK) the most abundant substrate available for DFR. This would agree with Kuehnle *et al.* (1997), wherein the chemical analysis of *Den.* x Icy Pink 'Sakura' detected only kaempferol derivatives as the major flavonols, with no detectable levels of

3'- hydroxylated quercetin derivatives, suggesting very low or no activity of F3'H in flowers (Kuehnle *et al.*, 1997).

Breeding efforts to create a wide variety of novel and desirable flower colors have been highly successful in some species (e.g. *Impatiens*). However, for other species the range of colors is limited because of specific deficiencies or features of the anthocyanin biosynthetic pathway (e.g. violet in carnation and orange in petunia). In this work, we cloned a *Dendrobium Dfr* gene and showed that this DFR can efficiently reduce all three substrates; DHK, DHQ, and DHM to produce pelargonidin, cyanidin, and delphinidin in the transgenic petunia. Our results conclude that the lack of pelargonidin type orange-colored *Dendrobium* flowers is not due to the substrate specificity of its DFR.

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CHAPTER 4

CHARACTERIZATION OF *MUT11*, A POTENTIAL GENE FOR RESISTANCE TO CymMV

4.1 INTRODUCTION

There are numerous potexviruses affecting many different crops worldwide. *Cymbidium mosaic virus* (CymMV) is the most important potexvirus of orchids, causing significant yield losses (Dunn, 1980; Wisler, 1989; Hu and Ferreira, 1994). Foliar symptoms of CymMV include chlorotic to necrotic, spots, and sunken patches, although infected plants can be symptomless as well (Lawson and Brannigan, 1986), making it difficult to diagnose and control the spread of virus. Floral symptoms are color breaking in flower, premature flower necrosis, or browning during shipment (Lawson and Brannigan, 1986; Wisler, 1989). Moreover, it also reduces plants vigor and decreases production yield. Chemical abatement of the spread of this potexvirus is an option (Hu *et al.*, 1994 paper) and other means for control and eradication have been described (Kuehnle, 1996). Genetic resistance offers the best means for eliminating this problem for orchid growers.

Systemic infection of orchids by CymMV entails translocation from the infection site to the adjacent cells and then to distal tissues using virus-expressed movement proteins (MPs) and coat protein (Carrington *et al.*, 1996; Beck *et al.* 1991). CymMV movement requires proteins encoded by three overlapping open reading frames that form

a triple-gene block (TGB). These proteins are designated TGB1, TGB2 and TGB3 according to the position of each gene (Solovyev *et al.* 1996) and are of sizes 26 kDa, 13 kDa, and 10 kDa, respectively (Wong et al., 1997). CymMV thus shares the TGB genome organization, essential for virion movement, with the other potexviruses as well as carlaviruses, hordeiviruses and some furoviruses (Morozov *et al.*, 1989; Foster *et al.*, 1988; Huisman *et al.*, 1988; Skryabin *et al.*, 1988; Rupasov *et al.*, 1989). Mutational analyses of infectious cDNA clones of virus genomes indicated that all three TGB proteins are essential for the virus movement process (Petty and Jackson, 1990; Beck *et al.*, 1991; Gilmer *et al.*, 1992; Herzog *et al.*, 1998) and that MPs combined with CP are needed for long-distance spread of CymMV within the plant (Ajjikuttira *et al.*, 2005). Use of plants transgenic for the MP has provided resistance to several viruses (Doem *et al.*, 1990; Lapidot *et al.*, 1993; Beck *et al.*, 1994; Cooper *et al.*; 1995; Seppanan *et al.*, 1997; Borth *et al.*, in press).

Our early work in the model plant *N. benthamiana* showed that a CymMV movement protein strategy, but not a coat protein strategy, was effective in preventing systemic infection by CymMV. Results showed that 5 of 9 transgenic *N. benthamiana* lines transformed with a six single-base-pair mutated movement protein gene (*Mut11*) were resistant to CymMV, but only 1 of 27 lines with the CymMV coat protein constructs showed high resistance compared to the virus-inoculated untransformed control (Borth *et al.*, in press). The coat protein strategy was also ineffective in transgenic *Dendrobium* when challenged with high viral titre (Chia *et al.*, 1992). Therefore, the aim of our subsequent research was to develop a CymMV resistant *Dendrobium* orchid using the

gene for the same point-mutated movement protein, *Mut11*, in an orchid gene expression vector. This was accomplished over a six year period using *Dendrobium* genetic engineering protocols developed and reported previously by our group (reviewed in Kuehnle 1997; Mudalige and Kuehnle 2004).

4.2 OBJECTIVE

This research was focused on characterizing the molecular genetic and virus resistant properties of *Dendrobium* plants that contain a CymMV cDNA clone encoding a movement protein with a site - specific mutation, *Mut11*. The resistance vector encodes the CymMV movement protein TGB2 (14k) and TGB3 (10k) under the control of a truncated ubiquitin3 (UBQ3) promoter region from *Arabidopsis thaliana*. Transgenic plants were produced via particle bombardment.

4.3 MATERIALS AND METHODS

4.3.1 Plant Materials and Culture Media

Two commercial *Dendrobium* cultivars, *D.* x Jaquelyn Thomas 'Uniwai Mist' (UH800) and *D.* x Jaq - Hawaii 'Uniwai Pearl' (UH306), were used in this study. PLBs were propagated from excised apical and lateral buds in liquid modified VW medium (Vacin and Went, 1949) containing 2 % (w/v) sucrose, 15 % (v/v) coconut water, and 57 mg L^{-1} iron chelate (Sequestrene 330 Fe), pH 5.2. All tissues cultures were grown at 25 $\pm$ 2° C under illumination with a 16-h photoperiod of 40 µmol m^{-2} sec^{-1} photon flux density provided by cool white and Gro-lux Sylvania fluorescent lamps (GTE Corp., Danvers, MA).

4.3.2 *Mut11* Gene, Plasmid DNA and Particle Bombardment

The *Mut11* gene derived from a sense orientation of the CymMV movement protein (Hawaiian isolate) with six single base-pair changes was produced and designed as described in Borth *et al.* (in press). The *Mut11* was prepared by PCR using template DNA synthesized by RT-PCR of the 14K and 10K regions of the triple-gene block sequence of CymMV. Primers used for PCR amplification of the CymMV MP gene were determined based on comparisons of published sequence information (Neo *et al.*, 1992, 1993; Barry *et al.*, 1996). The amplification primers P1 and P2 recognized sequences from bases 5002–5027 and 5464–5487 and contained *Nco*I sites at their 5′ ends (Figure 4.1 A). Two mutagenesis primers, P3 and P4, nested between primers P1 and P2, shared 31 bases of sequence homology (Fig. 4.1A) and incorporated six single-base changes to the CymMV-H isolate sequence. These two primers were used in combination with primers P1 and P2 to produce two amplicons (P1/P3 and P2/P4) that spanned the CymMV viral 14K and 10K regions, respectively. These mutated amplicons were then used together as templates in another PCR with primers P1 and P2 to produce a full-length copy of the 14K and 10K region of the triple gene block that incorporated the six base-pair mutation and contained *Nco*I sites at each terminus (Fig. 4.1 B; Borth *et al.*, in press). This mutated amplicon in pBI525 was digested with *Nco*I and blunt end cloned into pSAN150 (courtesy of Sanford Scientific Inc.), putting the *Mut11* under control of the ubiquitin3 (UBQ3) promoter from *Arabidopsis thaliana*. However, the UBQ3 promoter that we used are intronless, which might be less expression level 2.5-3 times than the one with the intron (Norris *et al.*, 1993).

Vector pSAN150:*Mut11* was used for biolistic co-transformation with pSAN154 (courtesy of Sanford Scientific Inc.), containing a selectable marker gene *hpt*II (hygromycin phosphotransferase) under the control of UBQ3 promoter (Table 4.1). All constructed plasmids were transferred into *Escherichia coli* DH5α and plasmid DNA was extracted using plasmid DNA isolation miniprep or midiprep kits (Qiagen, Valencia, CA). Plasmids containing *Mut11* or the selectable marker gene were coated onto 1.6 μm gold particles (Bio-Rad, Hercules, CA) in a 1: 1 (w/w) ratio, using the procedure of Nan and Kuehnle (1995). Microprojectile bombardments were carried out using Bio-Rad model PDS-1000 / He Biolistic® particle delivery system (Bio-Rad, Hercules, CA) at a vacuum pressure of 26-28 mm Hg, rupture disc pressure of 1100 psi, and target tissue placed on the 4th stage from the top using the standard laboratory protocol (Nan and Kuehnle, 1995). Plant materials in each individual plate received two shots. Plates with shot PLBs were kept under darkness for two days. All plant tissues were placed on growth media (VW liquid) for approximately two weeks without selection and subsequently transferred onto selection medium.

Table 4.1 Different plasmid constructs for *Mut11*, a mutated CymMV movement protein gene, and selectable marker *Hpt* for hygromycin phosphotransferase with corresponding regulartory elements used for particle bombardment into orchid tissues.

Plasmid ID	Gene	Promoter	Terminator	Purpose
pSAN150: *Mut11*	*Mut11*	Intronless UBQ3	*nos*	Interrupt the movement of Cymbidium Mosaic Virus
pSAN154	*Hpt*	Intronless UBQ3	*nos*	Selectable marker for screening transformed plants on hygromycin

A.

P1: CATCGACCATGGCAGGCTTAGTTCCA
P2: CTCTCACCATGGCTCCCATGATTATTTCAAGTTATT
P3: GTAGTTTATCTGCTTGG*C*A*G*CG*G*CTTTA*A*ATTGG*G*CG*G*CGGAGGGG
AAGCGGT
P4: CTCCG*C*CG*C*CCAAT*T*TAAAG*C*CG*C*T*G*CCAAG

B.

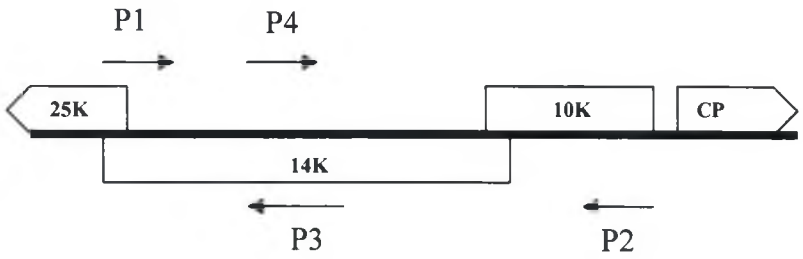

Figure 4.1 Primers used to construct mutant movement protein gene, *Mut11*, from CymMV - H. A. Sequences of primers are in sense orientation with the 5′ end to the left. The *Nco*I site in primers P1 and P2 is underlined. The regions of sequence homology between primers P3 and P4 are underlined. The bold italic letters indicated the six bases changed from CymMV – H. B. Diagram of the relative locations of PCR primers used to generate the mutant movement protein gene, *Mut11*, from CymMV (Borth *et al.*, in press).

4.3.3 Selection and Regeneration of Transformants

Different levels of hygromycin B (Sigma, St. Louis, MO) ranging from 0 – 25 $mg\,L^{-1}$ were used for selection of PLBs of UH800 and UH306 co-bombarded with pSAN150. Selection regimes used for each bombardment are listed with details in Table 4.2. Plantlets were regenerated from PLBs on banana medium consisting of VW medium supplemented with 75 g of blended flesh banana and solidified with 7 $g\,L^{-1}$ agar in 10 cm x 2.5 cm disposable Petri plates. Growing plantlets were transferred into Magenta G7 boxes for further growth while keeping track of the plate number and the PLB number of each plantlet.

4.3.4 Selection Putative Transgenic Plants by PCR Analysis

Leaves from regenerated plantlets in a single Magenta box were cut and pooled. One gram of leaves was arbitrarily taken to extract genomic DNA using DNeasy® Plant Minikit (Qiagen, Valencia, CA) following the manufacturer's instructions. Primer sequences and PCR conditions used for detection are summarized in Table 4.3. All PCR reactions were carried out with 200 – 250 ng of genomic DNA, Red Taq DNA polymerase (Sigma, St. Louis, MO), 0.4 µM primer concentration and 0.2 mM dNTP concentration in an iCycler thermal cycler (Bio-Rad, Hercules, CA). After that, plants from a single Magenta box that showed positive PCR results were separated and cultured as four individual plants per Magenta box. One gram of leaves from each individual plant was removed to extract genomic DNA and PCR was performed to confirm results as described above.

Table 4.2 Time table of co-bombardment, selection, plant recovery and analysis of two cultivars, *D.* x Jaquelyn Thomas 'Uniwai Mist' (UH800) and *D.* x Jaq – Hawaii 'Uniwai Pearl' (UH306) PLBs

Constructs and Date of Bombardment	# of[a] Plates	Selection Regime	Transfer to Regeneration Medium	% Survival of PLBs[b]	Status of the experiment
UH800+pSAN154 PSAN150/Mut11 02/07/00	12	10 days in liquid, no selection 14 days in liquid with hygromycin 12.50 mg·L^{-1}	3/3/00	9.5% (40/420) 9/14/00	PCR^{+} plants were potted on 12/14/02. Fully established plants were challenged with CymMV to investigate the resistant in transgenic plants on 6/14/03. TBIA was used to accesses the infection spread of CymMV. The plants that showed negative results from TBIA were brought to the lab to do RT-PCR to access the expression of *Mut11* on 1/14/04
None 02/07/00	2	10 days in liquid, no selection 14 days in liquid with hygromycin 12.50 mg·L^{-1}	3/30/00	2.8% (2/70) 9/14/00	
None 02/07/00	1	24 days in liquid, no selection	3/30/00	94.2% (33/35) 9/14/00	
UH306+pSAN154 pSAN150/Mut11 11/16/99	9	10 days liquid, no selection 14 days in liquid with hygromycin 20.0 mg·L^{-1}	12/20/99	6.03% (10/315) 6/24/00	
None 11/16/99	2	10 days liquid, no selection 14 days in liquid with hygromycin 12.5 mg·L^{-1}	12/20/99	1.43% (1/70) 6/24/00	
None 11/16/99	2	24 days in liquid, no selection	12/20/99	72.8% (51/70) 6/24/00	

[a] 35 PLBs per plate
[b] Number of PLBs producing plantlets after antibiotic selection out of total number bombarded

Table 4.3 Primer sequences and PCR conditions used in amplification of the inserted genes from transgenic *Dendrobium* x Jaquelyn Thomas 'Uniwai Mist' (UH800) and *Dendrobium* x Jaq – Hawaii 'Uniwai Pearl' (UH306).

Primer ID	Sequence	Gene Amplified	PCR Condition
HyG– 5′ HyG –3′	AAGTTCGACAGCGTCTCCGAC-5′ TTCTACACAGCCATCGGTCCA-3′	*hpt*II	95°C – 4 min. (95°C – 1 min, 61°C – 1 min, 72°C – 2 min) x 40 cycles 72°C – 8 min. 2.3mM $MgCl_2$ concentration, Red Taq (Sigma)
Mut11-5′ series I Mut11-3′ series I	CTCTCACCATGGCTCCCATGATTATTTCAAGTTATT-5′ CATCGACCATGGCAGGCTTAGTTCCA-3′	Mutated Movement Protein	95°C – 15 min (94°C – 1 min, 55°C – 1 min) x 40 cycles 55°C – 5 min. 0.5 mM $MgCl_2$ concentration, Red Taq (Sigma)
Mut11-5′ series II Mut11-3′ series II	GCCACTTGTAGTCAGGATATTTCG-5′ GGCTTTTGGTTTGCGATATAAAG-3′	Mutated Movement Protein	95°C – 2 min (94°C – 40 sec, 53°C – 40 min, 72°C – 1 min) x 30 cycles 72°C – 10min. Platinum® PCR SuperMix (Invitrogen)

4.3.5 Characterization of Transgenic Plants

Transgenic *Dendrobium* UH800 and UH360 plants were transitioned into the greenhouse and potted up in community pots for six months and transplanted to 3 inch pots for one to two years until fully established at the Magoon greenhouse, University of Hawaii at Manoa. Plants were challenged with CymMV using standard mechanical inoculation techniques (Fig. 4.2). CymMV inoculum was prepared from a CymMV-infected *Dendrobium* by grinding 1 g of fresh leaf tissue in 10 mL buffer (0.1 m KH_2PO_4, 0.01 M $MgCl_2$; pH 7.2). The inoculum concentrations at 1: 10 and 1: 1000 dilutions were used. Scoring for virus infection and movement used the Tissue Blot Immunoassay protocol (TBIA) of (Borth *et al.*, in press). TBIA was measured after inoculation for 1 week, 2 weeks, 1 month, 2 months, 3 months, and 6 months.

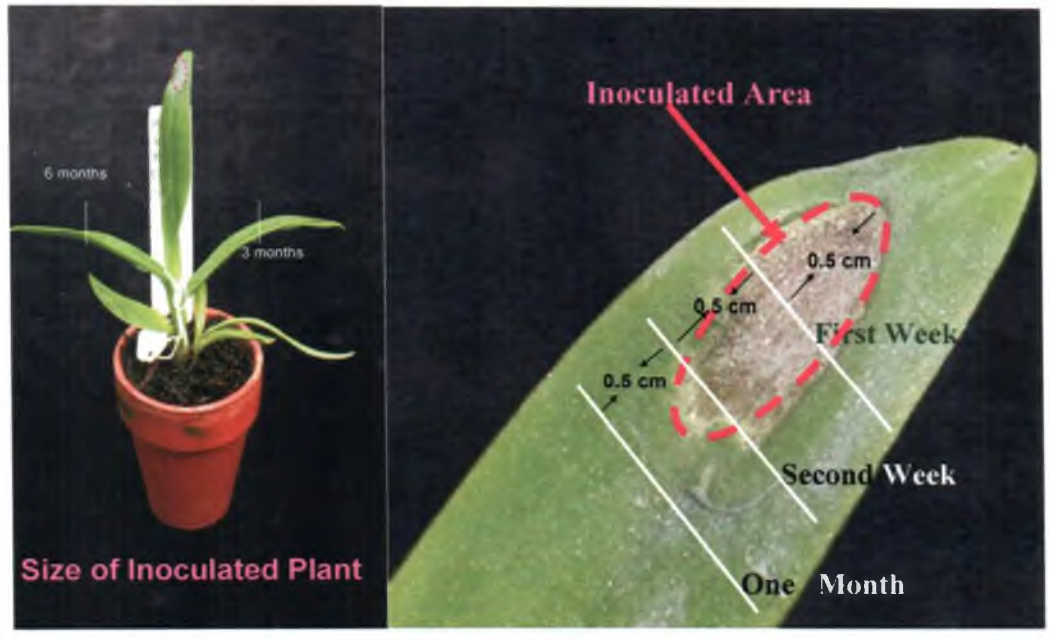

Figure 4.2 CymMV inoculated *Dendrobium* plants showing oval inoculation region and three sampling sites for leaf Tissue Blot Immunoassay.

4.3.6 RT-PCR Analysis

Those plants that were positive for the *Mut11* gene by PCR analysis and showed negative for virus by TBIA analysis were chosen for further molecular evaluation. RNA from leaves was extracted using the method of Champagne and Kuehnle (2000). An aliquot containing 5 μg of total RNA was treated with 1 unit of DNase I (Ambion Inc., Austin, TX) in 1X DNase I buffer in a 10 μL volume at 37°C for 20 minutes, according to the manufacturer's protocol. The activity of DNase was stopped by adding 1.1 μL of DNase inactivating reagent provided with the enzyme. Complementary DNA (cDNA) was synthesized from 5 μg of total RNA using 100 units of SuperScript® II RNase H reverse transcriptase (Invitrogen, Carlsbad, CA) in 1X first strand synthesis buffer (50 mM Tris-HCl, 75 mM KCl, 3mM $MgCl_2$, pH 8.3), supplemented with 0.01M DTT and 0.5 mM dNTPs, by incubating the reaction mixture at 42°C for 50 minutes. Oligo dT (T_{20}-T7) primer was used to prime the first strand cDNA synthesis. The reaction was stopped by incubation of the mixture at 70°C for 15 min. RNA template was removed by incubating the reaction mixture with 2 units of RNase H (Promega, Madison, WI) at 37°C for 20 minutes. The RNase H enzyme was inactivated by incubation at 60°C for 10 min. Sample was stored at -20°C in preparation for PCR amplifications. PCR conditions are shown in Table 4.3.

4.3.7 Southern Analysis of transgenic *Dendrobium*

a) Restriction endonuclease digestion and electrophoresis of genomic DNA restriction fragments.

Genomic DNA obtained using the protocol for co-isolating DNA from horticultural *Aroids* (Aragon *et al.*, 2004) was digested in a 250 µL reaction containing 15 µg gDNA and 20 units of restriction enzyme per µg of DNA, in a 1X enzyme reaction buffer supplemented with 1X Bovine Serum Albumin (BSA)(New England Biolabs, Beverly, MA) and 4 mM spermidine. Reactions were incubated at 37 °C for 16 hr. Following digestion, 0.1 volume of sodium acetate (pH 5.5) and two volumes of absolute ethanol were added to each reaction and the mixture incubated at -20°C for 30 min. The reaction was subsequently centrifuged at 13,000 rpm for 30 min, the supernatant removed, and the pellet washed 2X in 70% (v/v) ethanol. DNA pellets were air-dried and resuspended in 50 µL 10 mM Tris buffer (Tris-HCl, pH 8.0). Prior to electrophoresis, digested DNA was heated to 65°C for 10 min and then transferred to ice. After addition of one-sixth volume loading dye, samples were allowed to settle in the wells for 30 min. before initiating electrophoresis. Size fractionated, digested DNA was visualized by UV illumination.

b) Transfer of digested genomic DNA to nylon membrane

After electrophoresis, gels were soaked in depurination solution (0.25 N HCl) for 10 min and thoroughly rinsed in 3X distilled water. The rinsed gel was transferred to denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 2 x 15 min washes at room temperature and then to neutralization solution (0.5 M Tris-HCl pH 7.0, 1.5 M NaCl) for 2 x 15 min washes. Digested genomic DNA was transferred from the neutralized gels to a positively charged nylon membrane (Schleicher & Schuell, BioScience) following the manufacturers manual. Transfer was allowed to proceed for 10 h after which the

membrane was soaked in 5X SSC, pH 7.0 (0.6 M NaCl, 0.06 M Na citrate) for 5 min. The transferred DNA was fixed using optimal UV crosslink (Spectrolinker model XL-100 UV crosslinker).

c) Synthesis of ^{32}P labelled probe

^{32}P-labelled DNA probed was prepared using the *Mut11* sequence by random-priming using the Prime a Gene Kit (Promega, Madison, WI).

d) Hybridization and post - hybridization

Blots were prehybridized and hybridized with the probes (2 x 10^6 cpm/ml) at 65°C (Church and Gilbert, 1984).

e) Detection of ^{32}P - labelled probe

After washing, the membrane was sealed between plastic sheets, placed in an autoradiograph cassette and exposed to Biomax MS film (Eastman Kodak, Rochester, NY). The cassette was initially incubated at -80°C for 40 h. and film was developed to observe the hybridization signal.

4.3.8 Confirmation of transgenic plants by PCR primer series II

Leaves were sampled from nine individual plants that continued to remain free of CymMV after three sequential inoculations with CymMV. Genomic DNA was extract from 100 $mg \cdot L^{-1}$ samples using the Nucleon Phytopure plant DNA extraction kit (Amersham Biosciences Crop, NJ) following the manufacturer's protocols. Primer series II sequences that contained the promoter region in the forward primer and corresponding PCR conditions used for detection are summarized in Table 4.3. All PCR reactions were

carried out with 200 – 250 ng of genomic DNA, 1 µl each of 20 mM forward and backward primers, and 45 µl of Platinum® PCR SuperMIX (Invitrogen, CA) with the volume adjust to 50 µl by adding sterile distilled water.

4.3.9 Sequence Analysis

The PCR products from transgenic plants that still remained free from CymMV, were amplified by primer series II (Table 4.3) and were separated on agarose gels in 1X TAE buffer (40 mM Tris-acetate, 1 mM Na_2EDTA). Amplified DNA fragments with expected molecular weight were excised and purified using MiniElute™ Gel Extraction Kit (Qiagen) and ligated into PGEM®-T easy vector (Promega) following the manufacturers' protocols. The ligated plasmids were transformed to competent *Escherichia coli* DH5α strain. PCR products were sequence by using M13 Forward and M13 Reverse primers at the Greenwood Molecular Biology Facility of the University of Hawaii.

4.4 RESULTS

4.4.1 Selection and Regeneration

There was a variation in different selection regimes as shown in Table 4.4. Hygromycin was used in liquid growth media for selecting tolerance of two different *Dendrobium* hybrids. The result from the kill curve of UH800 showed that selection media with 10 mg L^{-1} hygromycin killed 65% of PLBs, whereas 15 mg L^{-1} hygromycin killed 75% of PLBs. An intermediate concentration of 12.5 mg L^{-1} hygromycin was chosen for selecting transformed plants. A 31.25% survival rate was obtained from this selection protocol. For UH306, we used 20 mg L^{-1} hygromycin (A. Kuehnle, unpublished results) for selecting transformed plants.

4.4.2 PCR Analysis

The individual plants used for DNA extraction were three to five centimeters tall (Fig. 4.3). Two sets of transgenic plants were analyzed for the inserted *Mut11* gene. The first set was UH800 bombarded with pSAN150:*Mut11* and pSAN154 and analyzed for the *Mut11* as well as the selectable marker, *hpt*. An example of an ethidium bromide stained gel photograph of PCR product of the *Mut11* gene is shown in Figure 4.4. Genomic DNA isolated from a non-transformed plant and sterile water without DNA, were used as negative controls. The plasmid DNA (pSAN150: *Mut11*) and/or genomic DNA from a known PCR-positive plant was used as positive controls. From 153 individual UH800 plants tested, 34 individual plants were positive for both genes while 25 individual plants did not contain either of the genes (Table 4.5). There were 29 individual plants positive for *Mut11* gene only, while 26 individual plants were positive

only for the selectable marker gene (Table 4.5). In summary, 26/153 individual UH800 plants contained the selectable marker gene and 63/153 individual UH800 plants contained the *Mut11* gene (Table 4.7).

The second set of plants was UH306 bombarded with the same plasmids was analyzed for the *Mut11* as well as the selectable marker (*hpt*II). An example of an ethidium bromide stained gel photograph of PCR product of the *Mut11* gene is demonstrated in Fig. 4.4. From 106 individual plants tested, 17 individual UH306 plants were positive for both genes, whilst 13 individual UH306 plants contained neither of the genes (Table 4.6). There were 12 individual UH306 plants positive for *Mut11* gene only, while 20 individual plants were positive only for the selectable marker gene (Table 4.6). In summary, 20/106 individual UH306 plants contained the selectable marker gene 29/106 individual UH306 plants contained the *Mut11* gene (Table 4.7).

Table 4.4 Kill curve for *Dendrobium* x Jaquelyn Thomas 'Uniwai Mist' (UH800) after treated with hygromycin for 2 weeks.

Hygromycin Conc.	No. of PLBs	% Survival of PLBs	% Kill
0	40	100	0
5	40	95	5
10	40	35	65
15	40	25	75
20	40	0	100
25	40	2.5	97.5

Figure 4.3 Regenerated plantlets of *Dendrobium* x Jaquelyn Thomas 'Uniwai Mist'(UH800) and *Dendrobium* x Jaq – Hawaii 'Uniwai Pearl'(UH306) regenerated on banana medium lacking antibiotic after two weeks of hygromycin selection. Individual plants were sampled to test for the presence of the inserted genes using PCR.

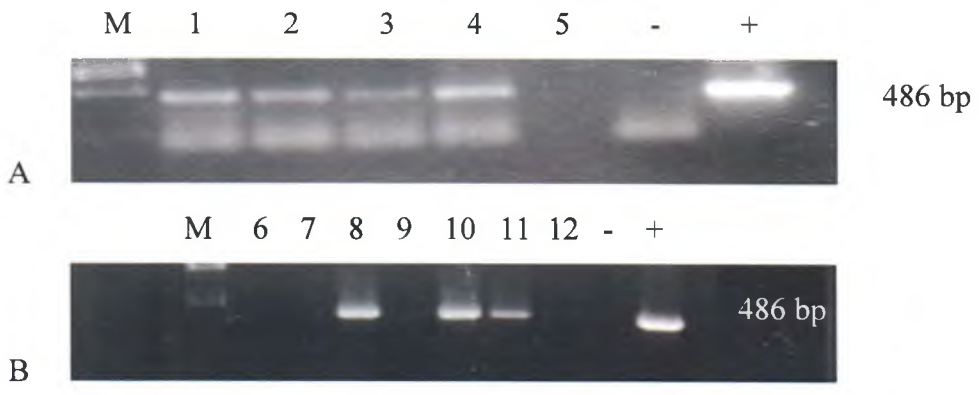

Figure 4.4 Agarose gel electrophoresis of PCR amplified products of *Mut11* gene from bombarded *Dendrobium* x Jaquelyn Thomas 'Uniwai Mist'(UH800) (A) and *Dendrobium* x Jaq – Hawaii 'Uniwai Pearl'(UH306) (B). Primers specific for *Mut11* gene were used to amplify a 486 bp fragment from genomic DNA of UH800 and UH306 regenerated from PLBs co-bombarded with pSAN150: *Mut11* and pSAN154. Lane m = molecular weight markers; lanes 1-5 = genomic DNA from some pooled samples of UH800; lanes 6-12 = genomic DNA from some pooled samples of UH306; lane - = genomic DNA from a control plant that was not bombarded with *Mut11*; lane = plasmid DNA from *Mut11* plasmid.

Table 4.5 Results of PCR experiments to detect the presence of *Mut1 1* and *hpt* genes from bombarded and selected plants of *Dendrobium* x Jaquelyn Thomas 'Uniwai Mist'(UH800).

DNA sample	*Mut1 1*[a]	Hyg[b]	Potting date and status
UH800-1	-	n.d	Discarded
UH800-2	-	n.d	Discarded
UH800-3	-	n.d	Discarded
UH800-4	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-5	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-6	-	n.d	Discarded
UH800-7	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-8	-	n.d	Discarded
UH800-9	-	n.d	Discarded
UH800-10	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-14-1	-	n.d	Discarded
UH800-14-2	-	n.d	Discarded
UH800-14-3	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-14-4	-	n.d	Discarded
UH800-14-5	-	n.d	Discarded
UH800-14-6	-	n.d	Discarded
UH800-14-7	+	+	Died after moved to greenhouse
UH800-14-8	-	n.d	Discarded
UH800-14-9	+	+	Died after moved to greenhouse
UH800-18-a	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-18-b	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-24	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-1-A	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-1-B	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-1-C	+	+	Died after moved to greenhouse
UH800-1-D	+	-	Died after moved to greenhouse
UH800-1-E	+	+	Died after moved to greenhouse
UH800-1a-1	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-1a-2	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-1a-3	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-1a-4	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-1a-5	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-1a-6	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-1a-7	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-3a-1	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-1bI-a	-	n.d	Discarded
UH800-1bI-b	-	n.d	Discarded
UH800-1bI-c	-	n.d	Discarded
UH800-1bI-e	-	-	Discarded
UH800-1bI-f	-	-	Discarded
UH800-1bI-g	-	-	Discarded
UH800-1bI-h	-	-	Discarded
UH800-1bI-I	-	-	Discarded
UH800-1bI-j	-	-	Discarded
UH800-1bI-k	-	+	Discarded

Table 4.5 (Continued) Results of PCR experiments to detect the presence of *Mut11* gene and *hpt* gene from bombarded and selected plants of *Dendrobium* x Jaquelyn Thomas 'Uniwai Mist'(UH800).

DNA sample	*Mut11*[a]	Hyg[b]	Pot date and status
UH800-1bI-1	-	-	Discarded
UH800-1bII-a	-	n.d	Discarded
UH800-1bII-b	-	n.d	Discarded
UH800-1bII-c	-	-	Discarded
UH800-1bII-d	-	-	Discarded
UH800-1bII-e	-	-	Discarded
UH800-1bII-f	-	n.d	Discarded
UH800-1bII-g	-	-	Discarded
UH800-1bII-h	-	-	Discarded
UH800-1a	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-2a	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-1b	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-2b	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-3b	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-2bII	-	+	Discarded
UH800-3a	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-3bI	-	-	Discarded
UH800-3bII	-	-	Discarded
UH800-3bIII	-	-	Discarded
UH800-1c	+	+	Died after moved to greenhouse
UH800-3c	-	-	Discarded
UH800-4a-1	-	-	Discarded
UH800-4a-2	-	+	Discarded
UH800-4a-3	-	-	Discarded
UH800-4a-4	-	+	Discarded
UH800-4a-5	-	n.d	Discarded
UH800-4a-6	-	n.d	Discarded
UH800-4a-7	-	n.d	Discarded
UH800-4a-8	-	-	Discarded
UH800-4a-9	-	+	Discarded
UH800-4a-10	-	+	Discarded
UH800-4a-11	-	+	Discarded
UH800-5A-12	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-5I	-	-	Discarded
UH800-5II	-	n.d	Discarded
UH800-5III	-	n.d	Discarded
UH800-6a	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-6bI-a	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-6bI-b	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-6bI-c	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-6bI-d	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-6bI-e	-	+	Discarded
UH800-6bI-f	-	+	Discarded
UH800-6bI-g	-	+	Discarded
UH800-6bI-h	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01

Table 4.5 (Continued) Results of PCR experiments to detect the presence of *Mut1 1* gene and *hpt* gene from bombarded and selected plants of *Dendrobium* x Jaquelyn Thomas 'Uniwai Mist'(UH800).

DNA sample	*Mut1 1*[a]	Hyg[b]	Pot date and status
UH800-6bI-I	-	n.d	Discarded
UH800-6bI-j	-	n.d	Discarded
UH800-6bI-k	-	n.d	Discarded
UH800-6bI-l	-	-	Discarded
UH800-6bI-m	-	+	Discarded
UH800-6bII-a	-	+	Discarded
UH800-6bII-b	-	+	Discarded
UH800-6bII-c	-	n.d	Discarded
UH800-6bII-d	-	n.d	Discarded
UH800-6bII-e	-	n.d	Discarded
UH800-6bII-f	-	n.d	Discarded
UH800-6bII-g	-	n.d	Discarded
UH800-6bII-h	-	n.d	Discarded
UH800-6bII-I	-	n.d	Discarded
UH800-6bII-j	-	n.d	Discarded
UH800-6bII-k	-	n.d	Discarded
UH800-6bII-l	-	-	Discarded
UH800-6bII-m	-	+	Discarded
UH800-6bII-n	-	+	Discarded
UH800-6bII-o	-	+	Discarded
UH800-6bII-p	-	n.d	Discarded
UH800-6bIII-a	-	n.d	Discarded
UH800-6bIII-b	-	n.d	Discarded
UH800-6bIII-c	-	n.d	Discarded
UH800-6bIII-d	-	+	Discarded
UH800-6bIII-e	-	-	Discarded
UH800-6bIII-f	-	+	Discarded
UH800-6bIII-g	-	+	Discarded
UH800-6bIII-h	-	+	Discarded
UH800-6bIII-I	-	+	Discarded
UH800-6bIII-j	-	+	Discarded
UH800-6bIII-k	-	-	Discarded
UH800-6bIII-l	-	+	Discarded
UH800-7a	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-7b	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-7c	-	+	Discarded
UH800-8	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-10	-	+	Discarded
UH800-11	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-12A-I	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-12b-a1	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-12b-a2	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-12b-a3	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-12b-a4	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-12b-b1	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01

Table 4.5 (Continued) Results of PCR experiments to detect the presence of *Mut11*gene and *hpt* gene from bombarded and selected plants of *Dendrobium* x Jaquelyn Thomas 'Uniwai Mist'(UH800).

DNA sample	*Mut11*[a]	Hyg[b]	Pot date and status
UH800-12b-b2	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-12b-b3	-	-	Discarded
UH800-12b-b4	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-12c	-	+	Discarded
UH800-13a	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-13b	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-14	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-15a-1	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-15a-2	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-15a-3	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-15a-4	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-15a-5	+	+	Died after moved to greenhouse
UH800-15a-6	+	-	Died after moved to greenhouse
UH800-15a-7	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-15a-8	+	-	Died after moved to greenhouse
UH800-15a-9	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-15a-10	+	-	Potted 1/18/01, Moved to greenhouse 4/20/01
UH800-15a-11	+	+	Potted 1/18/01, Moved to greenhouse 4/20/01

Table 4.6 Results of PCR experiments to detect the presence of *Mut11* gene and *hpt* gene from bombarded and selected plants of *Dendrobium* x Jaq – Hawaii 'Uniwai Pearl' (UH306).

DNA sample	*Mut11*[b]	Hyg[c]	Pot date and status
UH306-1	+	-	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-2	+	-	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-3	-	-	Discarded
UH306-4	-	+	Discarded
UH306-5	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-6	-	-	Discarded
UH306-7	-	+	Discarded
UH306-8	-	+	Discarded
UH306-9	+	-	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-A	-	n.d	Discarded
UH306-B	-	n.d	Discarded
UH306-C	-	n.d	Discarded
UH306-D	-	n.d	Discarded
UH306-E	-	n.d	Discarded
UH306-F	-	n.d	Discarded
UH306-G-1	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-H	-	n.d	Discarded
UH306-K	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-L	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-5I-a	-	n.d	Discarded
UH306-5I-b	-	n.d	Discarded
UH306-5I-c	-	n.d	Discarded
UH306-5I-d	-	n.d	Discarded
UH306-5I-e	-	+	Discarded
UH306-5I-f	-	+	Discarded
UH306-5I-g	-	-	Discarded
UH306-5I-h	-	-	Discarded
UH306-5I-I	-	+	Discarded
UH306-5I-j	-	+	Discarded
UH306-5II-a	-	n.d	Discarded
UH306-5II-b	-	n.d	Discarded
UH306-5II-c	-	n.d	Discarded
UH306-5II-d	-	n.d	Discarded
UH306-5II-e	-	n.d	Discarded
UH306-5II-f	-	n.d	Discarded
UH306-5II-g	-	n.d	Discarded
UH306-5II-h	-	n.d	Discarded
UH306-5II-I	-	n.d	Discarded
UH306-5II-j	-	-	Discarded
UH306-5III-a	+	-	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-5III-b	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-5III-c	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-5III-d	-	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-5III-e	-	n.d	Discarded

Table 4.6 (Continued) Results of PCR experiments to detect the presence of *Mut11*gene and *hpt* gene from bombarded and selected plants of *Dendrobium* x Jaq – Hawaii 'Uniwai Pearl' (UH306).

DNA sample	*Mut11*[b]	Hyg[c]	Pot date and status
UH306-5III-f	-	n.d	Discarded
UH306-5III-g	-	n.d	Discarded
UH306-5III-h	-	n.d	Discarded
UH306-5III-I	-	n.d	Discarded
UH306-5III-j	-	n.d	Discarded
UH306-5III-k	-	n.d	Discarded
UH306-5III-l	-	n.d	Discarded
UH306-5III-m	-	n.d	Discarded
UH306-7I-a	-	n.d	Discarded
UH306-7I-c	-	n.d	Discarded
UH306-7I-d	-	+	Discarded
UH306-7I-eI	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-7I-e2	+	-	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-7I-f	-	+	Discarded
UH306-7I-g	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-7II-a	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-7II-b	+	-	Died after moved to greenhouse
UH306-7II-c	+	-	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-7II-d	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-7II-e	-	n.d	Discarded
UH306-7II-f	+	+	Died after moved to greenhouse
UH306-7II-g	-	n.d	Discarded
UH306-7II-h	+	-	Died after moved to greenhouse
UH306-7III-a	-	+	Discarded
UH306-7III-b	-	-	Discarded
UH306-7III-c	-	+	Discarded
UH306-7III-d	-	-	Discarded
UH306-7III-e	-	+	Discarded
UH306-7III-f	-	+	Discarded
UH306-7III-g	-	n.d	Discarded
UH306-7III-h	-	+	Discarded
UH306-7III-I	-	-	Discarded
UH306-7III-j	-	n.d	Discarded
UH306-7III-k	-	n.d	Discarded
UH306-7III-l	-	n.d	Discarded
UH306-8-a	+	-	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-8-b	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-8-c	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-8-d	-	n.d	Discarded
UH306-8-e	-	n.d	Discarded
UH306-8-f	-	n.d	Discarded
UH306-8-g	-	n.d	Discarded
UH306-8-h	-	n.d	Discarded
UH306-8-I	+	-	Potted 1/22/01, Moved to greenhouse 5/1/01

Table 4.6 (Continued) Results of PCR experiments to detect the presence of *Mut1 1* gene and *hpt* gene from bombarded and selected plants of *Dendrobium* x Jaq – Hawaii 'Uniwai Pearl' (UH306).

DNA sample	*Mut1 1*[b]	Hyg[c]	Pot date and status
UH306-8-j2	+	-	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-8-k	+	-	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-8-l	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-8-I	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-9I-a	-	+	Discarded
UH306-9I-b	-	+	Discarded
UH306-9I-c	-	-	Discarded
UH306-9I-d	-	-	Discarded
UH306-9I-e	-	+	Discarded
UH306-9I-f	-	-	Discarded
UH306-9I-g	-	-	Discarded
UH306-9I-h	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01
UH306-9II-b	-	-	Discarded
UH306-9II-c	-	+	Discarded
UH306-9II-d	-	+	Discarded
UH306-9II-e	-	n.d	Discarded
UH306-9II-g	-	n.d	Discarded
UH306-9II-h	+	+	Potted 1/22/01, Moved to greenhouse 5/1/01

[a] PCR result for the *Mut1 1* gene. + = positive PCR result; - = negative PCR result; n.d.= not done.
[b] PCR result for the *hpt* gene. + = positive PCR result; - = negative PCR result; n.d.= not done.

Table 4.7 PCR analysis of *Dendrobium* x Jaquelyn Thomas 'Uniwai Mist' (UH800) and *Dendrobium* x Jaq – Hawaii 'Uniwai Pearl'(UH306).

Genotype	*Mut11* gene	Hyg gene	Both genes	Escape (non-transgenic)	PCR+/total plants analyzed
UH800	29/153 =18.9%	26/153 =16.9%	34/153 =22.2%	25/153 =16.3%	63/153
UH306	12/106 =11.3%	20/106 =18.8%	17/106 =16.0%	13/106 =12.3%	29/106

4.4.3 Production of transgenic Plants with CymMV Genes

After PCR analysis, the PCR positive transgenic orchid plants were removed from tissue culture and grown in the insect - proof cages in the greenhouse (Fig. 4.8). Plants were grouped according to their PLB origin. One group can have several lines of plants, which may or may not be independently transformed. There were twenty-four confirmed transgenic orchid plant groups (15 groups of UH800 and 9 groups of UH306) as shown by positive PCR tests for *Mut11* (Table 4.8). These fifteen independently transformed plant groups of cultivar UH800 and nine plant groups of cultivar UH306 were regenerated and tested for CymMV resistance.

Table 4.8 The 24 different transgenic *Dendrobium* orchid groups confirmed by PCR analysis.

Group	Line Designation
1	UH800II-15A-1, UH800II-15A -2, UH800II-15A -3, UH800II-15A -4, UH800II-15A -5, UH800II-15A -6, UH800II-15A -7, UH800II-15A -8, UH800II-15A -9, UH800II-15A -10, UH800II-15A -11
2	UH800-14-3, UH800-14-7, UH800-14-9, UH800-14-24
3	UH800-12B-A1, UH800-12B -A2, UH800-12B -A3, UH800-12B -A4
4	UH800-12B-B1, UH800-12B -B2, UH800-12B -B4
5	UH800-1-A, UH800-1 -B, UH800-1-D, UH800-1-E
6	UH800-6bI-A, UH800-6bI -B, UH800-6bI -C, UH800-6bI -D
7	UH800-4, UH800-5, UH800-7, UH800-10
8	UH800-1a, UH800-2a, UH800-3a, UH800-6a
9	UH800-1b, UH800-2b, UH800-3b
10	UH800-5A-12
11	UH800-12A-i
12	UH800-18-a, UH800-18-b
13	UH800-3a-1
14	UH800-6bI-H
15	UH800-1a-1, UH800-1a -2, UH800-1a -3, UH800-1a -4, UH800-1a -5, UH800-1a -6, UH800-1a -7
16	UH306-7II-A, UH306-7II -B, UH306-7II -C, UH306-7II -D, UH306-7II -F, UH306-7II -H
17	UH306-7I-E1, UH306-7I -E2, UH306-7I -G
18	UH306-8-A, UH306-8-B, UH306-8-C, UH306-8-D, UH306-8-J2, UH306-8-K, UH306-8-L
19	UH306-8I
20	UH306-L, UH306-K
21	UH306-5III-A, UH306-5III -B, UH306-5III -C
22	UH306-9II-H
23	UH306-1, UH306-2, UH306-5, UH306-9
24	UH306-G-1

4.4.4 Characterization of Transgenic Plants

Transgenic orchid plants were obtained and evaluated for CymMV resistance after mechanical inoculations in greenhouse experiments under controlled conditions. All of the plants of fourteen plant groups inoculated with a 1:10 dilution of CymMV became infected one month after inoculation (Table 4.9). The rest of nine groups were inoculated with 1:1000 dilution of CymMV. The result showed that six of nine plant groups transformed with the *Mut11* gene construct were negative in TBIA and thus free of virus six months after challenge with 1:1000 dilution of CymMV (Table 4.10).

From the inoculation test, results indicated that if the concentration of the inoculum was too high, none of the transgenic plants can survive. All transgenic orchid plants became infected by CymMV one month after inoculation (Table 4.9). The concentration of inoculum was decreased to a 1: 1000 dilution which is the minimum concentration that caused the local lesions in indicator plant (*Cassia occidentalis* L.) (J.S. Hu, unpublished data) and inoculated onto the rest of the PCR positive plants. Six different transgenic plant groups (17 plants) still remained free of CymMV six months after inoculation (Table 4.10).

4.4.5 RT-PCR Analysis

The six different transgenic plant groups that showed no virus in TBIA six months after challenge with 1:1000 dilution of CymMV were tested for the expression of the transgene, *Mut11*. RT-PCR analysis indicated that only two plants, UH800-1-B and UH306-7II-D, were positive of the inserted *Mut11* gene (Fig. 4.9). The other tested plants were negative for *Mut11* gene (Table 4.11). RNA samples were digested with

DNaseI prior to RT-PCR in order to remove all residual DNA, which might give a false positive on PCR.

Table 4.9 Result of tissue blot immunoassay after one month inoculated with 1:10 dilution of CymMV.

Genotype	TBIA Result	Genotype	TBIA Result	Genotype	TBIA Result
UH800-6bI-A	+	UH800-6bI-H	+	UH306-5	+
UH800-6bI-B	+			UH306-9	+
UH800-6bI-C	+	UH800-1a-1	+		
UH800-6bI-D	+	UH800-1a-2	+	UH306 (WT)	+
		UH800-1a-3	+	UH306 (WT)	+
UH800-4	+	UH800-1a-4	+	UH306 (WT)	+
UH800-5	+	UH800-1a-5	+		
UH800-7	+	UH800-1a-6	+	UH800 (WT)	+
UH800-10	+	UH800-1a-7	+	UH800 (WT)	+
UH800-24	+			UH800 (WT)	+
UH800-1a	+				
UH800-2a	+	UH306-8I	+		
UH800-3a	+				
UH800-6a	+	UH306-G-1	+		
		UH306-L	+		
UH800-1b	+	UH306-K	+		
UH800-2b	+				
UH800-3b	+	UH306-5III-A	+		
		UH306-5III-B	+		
UH800-12A-i	+	UH306-5III-C	+		
UH800-18-a	+	UH306-9II-H	+		
UH800-18-b	+				
		UH306-1	+		
UH800-3a-1	+	UH306-2	+		

Table 4.10 Result of tissue blot immunoassay after six months inoculated with 1:1000 dilution of CymMV.

Genotype	TBIA Result	Genotype	TBIA Result
UH800II-15A-1	-	UH800-1-E	Didn't survive transplanting
UH800II-15A-2	-		
UH800II-15A-3	-	UH800-5A-12	+
UH800II-15A-4	-		
UH800II-15A-5	Didn't survive transplanting	UH306-7II-A	+
UH800II-15A-6	Didn't survive transplanting	UH306-7II-B	Didn't survive transplanting
UH800II-15A-7	+	UH306-7II-C	+
UH800II-15A-8	Didn't survive transplanting	UH306-7II-D	-
UH800II-15A-9	-	UH306-7II-F	Didn't survive transplanting
UH800II-15A-10	-	UH306-7II-H	Didn't survive transplanting
UH800II-15A-11	+		
		UH306-7I-E1	+
UH800-14 -3	+	UH306-7I-E2	+
UH800-14 -7	Didn't survive transplanting	UH306-7I-G	+
UH800-14 -9	Didn't survive transplanting		
		UH306-8-A	+
UH800-12B- A1	+	UH306-8-B	+
UH800-12B-A2	+	UH306-8-C	-
UH800-12B-A3	-	UH306-8-D	-
UH800-12B-A4	-	UH306-8-J2	-
		UH306-8-K	-
UH800-12B-B1	-	UH306-8-L	-
UH800-12B-B2	+		
UH800-12B-B4	-	UH306 (WT)	+
		UH306 (WT)	+
UH800-1-A	Didn't survive transplanting	UH800 (WT)	+
UH800-1-B	-	UH800 (WT)	+
UH800-1-D	Didn't survive transplanting		

4.4.6 Tissue Blot Immunoassay Analysis (TBIA)

After RT-PCR analysis, all six groups of 17 plant lines (6 individual UH306 and 11 individual UH800) that showed negative results from TBIA and four individual wild type non-transgenic plants were re-inoculated with a 1: 1000 dilution of CymMV to confirm the resistance to CymMV. Then TBIA was performed 1 month after inoculation. Result showed that 14 plants out of 17 plants (UH800II-15A-1, UH800II-15A -2, UH800II-15A -3, UH800II-15A -4, UH800II-15A -9, UH800II-15A -10, UH800-12B-A3, UH800-12B-B4, UH800-1-B, UH306-8-C, UH306-8-D, UH306-8-J2, UH306-8-K, and UH306-8-L) were free of virus, whereas 4 non-transgenic yield types (2 of UH800 and 2 of UH306) were positive for virus (Table 4.12).

The plants which were free of virus were re-inoculated with 1: 1000 dilution of CymMV and analyzed by TBIA after 1 wk, 2 wk, 1 month, 3 month, and 6 months. Results showed that all six control plants and five individual transgenic plants (UH800-1-B, UH800II-15A-3, UH306-8-K, UH306-8-L, and UH306-8-D) showed positive for CymMV already two weeks after inoculation. Nine individual transgenic plants (UH800II-15A-1, UH800II-15A-2, UH800II-15A-3, UH800II-15A-4, UH800II-15A-9, UH800II-15A-10, UH800-12B-A4, UH800-12B-B4, and UH306-8-J2) still remained free from CymMV six months after inoculation (Table 4.13). From the first time inoculation with 1: 1000 dilution of CymMV until now, all these nine individual plants are free from virus more than two years.

Table 4.11 RT-PCR analysis results of six different transgenic orchid groups which were negative in tissue blot immunoassay six month after challenge with a 1: 1000 dilution of CymMV.

Genotype	TBIA Result	RT-PCR
UH800II-15A-1	-	-
UH800II-15A-2	-	-
UH800II-15A-3	-	-
UH800II-15A-4	-	-
UH800II-15A-9	-	-
UH800II-15A-10	-	-
.UH800-12B-A3	-	-
UH800-12B-A4	-	-
UH800-12B-B1	-	-
UH800-12B-B4	-	-
UH800-1-B	-	+
UH306-7II-D	-	+
UH306-8-C	-	-
UH306-8-D	-	-
UH306-8-J2	-	-
UH306-8-K	-	-
UH306-8-L	-	-

Table 4.12 Result of tissue blot immunoassay after one month re-inoculated with a 1: 1000 dilution of CymMV.

Genotype	TBIA Result	RT-PCR
UH800II-15A-1	-	-
UH800II-15A-2	-	-
UH800II-15A-3	-	-
UH800II-15A-4	-	-
UH800II-15A-9	-	-
UH800II-15A-10	-	-
.UH800-12B-A3	+	-
UH800-12B-A4	-	-
UH800-12B-B1	+	-
UH800-12B-B4	-	-
UH800-1-B	-	+
UH306-7II-D	+	+
UH306-8-C	-	-
UH306-8-D	-	-
UH306-8-J2	-	-
UH306-8-K	-	-
UH306-8-L	-	-
UH800 (WT)	+	Nd.
UH800 (WT)	+	Nd.
UH306 (WT)	+	Nd.
UH306 (WT)	+	Nd.

- = negative, + = positive, Nd = Not detemined

4.4.7 Southern Analysis of Transgenic *Dendrobium*

Southern analysis (section 4.3.7) was carried out on 9 individual putative *Mut11*-positive plants of *Dendrobium*.

To confirm the integration of *Mut11* genomes of the transgenic orchid plants, genomic DNA was digested with *Nco*I. The *Mut11* gene was excised from *Mut11* plasmid by using enzyme *Nco*I and labeled with ^{32}P. This probe was then used to evaluate the number of transgene integration sites.

Southern analysis of *Nco*I digested genomic DNA revealed that the *Mut11* gene was integrated in at least two individual *Mut11* plants (UH800II-15a-10 and UH800II-15a-1) as shown in Figure 4.5.

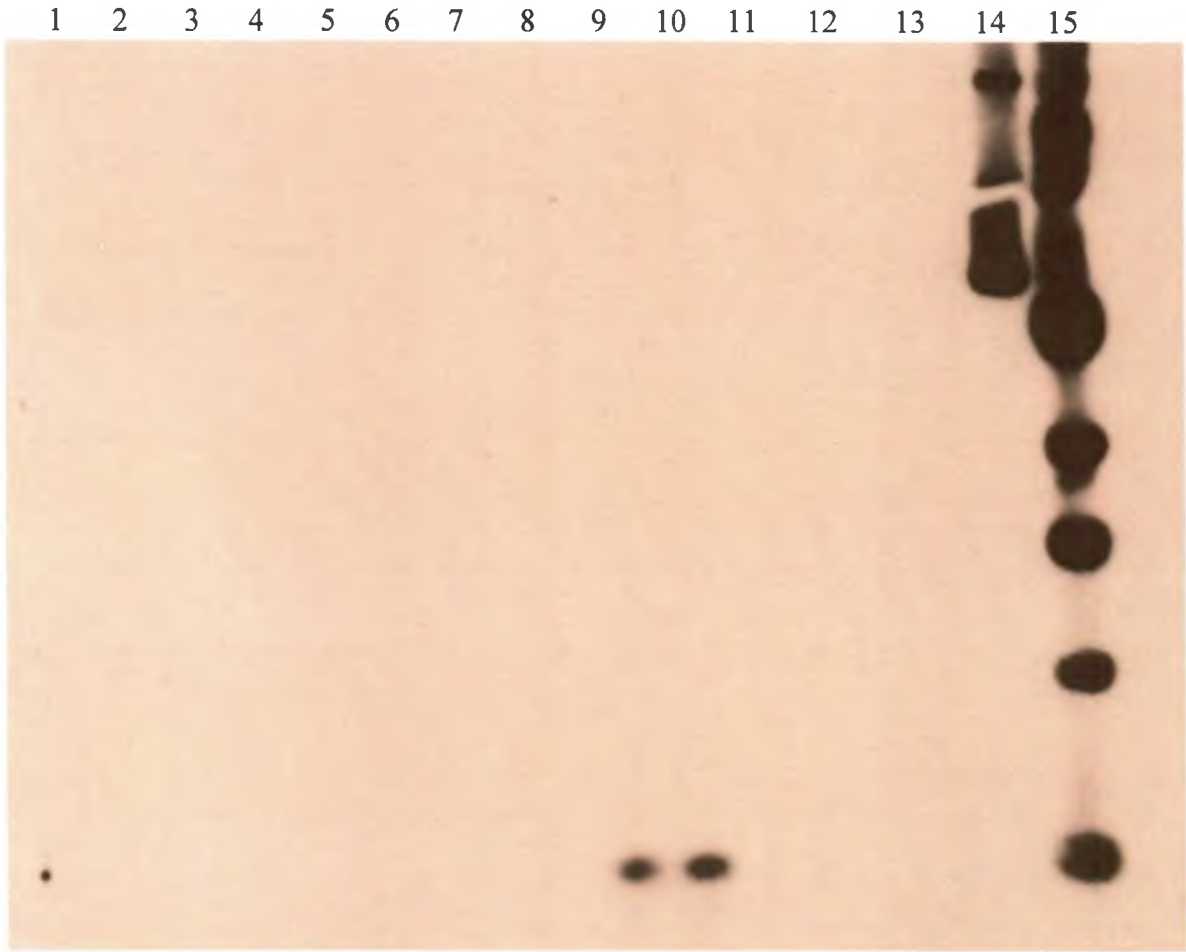

Figure 4.5 Autoradiographic for southern analysis of transgenic *Dendrobium* hybrids genomic DNA (15 μg) digested with *Nco* I visualized using a P^{32} labelled *Mut11* probe.

Lane 1 = UH306 (WT)
Lane 2 = UH800 (WT)
Lane 3 = UH800II-15a-4
Lane 4 = UH800-12b-a4
Lane 5 = UH800II-15a-3
Lane 6 = UH800II-15a-2
Lane 7 = UH306-8-j2
Lane 8 = UH306-8-j2
Lane 9 = UH800II-15a-10
Lane 10 = UH800II-15a-1
Lane 11 = UH800-12b-b4
Lane 12 = UH800-12b-a3
Lane 13 = UH800II-15a-9
Lane 14 = *Mut11* plasmid
Lane 15 = 1Kb Marker

Table 4.13 Tissue blot immunoassay analysis of orchid plants re-inoculated with a 1: 1000 dilution of CymMV after inoculated for 1 wk, 2 wk, 1 month, 3 months, and 6 months.

Genotype	Inoculation Date	TBIA 1 wk	TBIA 2 wk	TBIA 1 mo	TBIA 3 mo	TBIA 6 mo
UH800II-15A-1	3/1/04	-	-	-	-	-
UH800II-15A-2	3/1/04	-	-	-	-	-
UH800II-15A-3	3/1/04	-	-	-	-	-
UH800II-15A-4	3/1/04	-	-	-	-	-
UH800II-15A-9	3/1/04	-	-	-	-	-
UH800II-15A-10	3/1/04	-	-	-	-	-
UH800-12B-A4	3/1/04	-	-	-	-	-
UH800-12B-B4	3/1/04	-	-	-	-	-
UH800-1-B	3/1/04	+	-	+	+	+
UH306-8-D	3/1/04	-	-	+	+	+
UH306-8-J2	3/1/04	-	+	-	-	-
UH306-8-K	3/1/04	-	-	+	+	+
UH306-8-L	3/1/04	-	-	+	+	+
UH800 (WT)	3/1/04	+	+	+	+	+
UH800 (WT)	3/1/04	+	+	+	+	+
UH800 (WT)	3/1/04	+	+	+	+	+
UH800 (WT)	3/1/04	+	+	+	+	+
UH800 (WT)	3/1/04	+	+	+	+	+
UH800 (WT)	3/1/04	+	+	+	+	+

4.4.8 Confirmation of PCR analysis results using primer series II

Before PCR analysis, leaf samples of nine individual plants were analyzed for CymMV infection by Enzyme-Link Immunosorbent Assay (ELISA) at University of Hawaii Agriculture Diagnostic Service Center. Results indicated that eight plants out of the nine were free of CymMV 18 months after the third inoculation with 1: 1000 CymMV. The DNA from ten individual plants (two were virus infected plants) was used for PCR with primer series II to confirm the previous PCR analysis result. The forward primer was designed from the promoter region and the reverse primer was in the *Mut11* gene (Table 4.3). Results showed that all eight plants free from CymMV were transgenic for of *Mut11* by PCR analysis (Figure 4.6). In contrast, the two virus-infected plants lacked *Mut11* by this PCR analysis, even though these two plants were positive for PCR with primer series I. The forward and reverse primer of primer series I were designed from the *Mut11* region which is very similar to wild type CymMV triple gene block 2 (TGB2), since only 6 nucleotide were mutated from the wild type.

4.4.9 Sequence Analysis

To confirm the transgene is present in transgenic plants, three cloned PCR products amplified from both primer series I and series II from two different samples were sequenced. The nucleotide sequence of genomic DNA from transformed plants amplified by primer series I and primer series II are 100% similar to *Mut11* sequence as show in Figure 4.7A. These results indicated that the of *Mut11* transgene is present in transgenic orchids. According to six nucleotides different from TGB2 region of CymMV

(Hawaiian strain) wild type (Barry *et al.*, 1996) resulting in six amino acids changes in *Mut11* as show in Figure 4.7B.

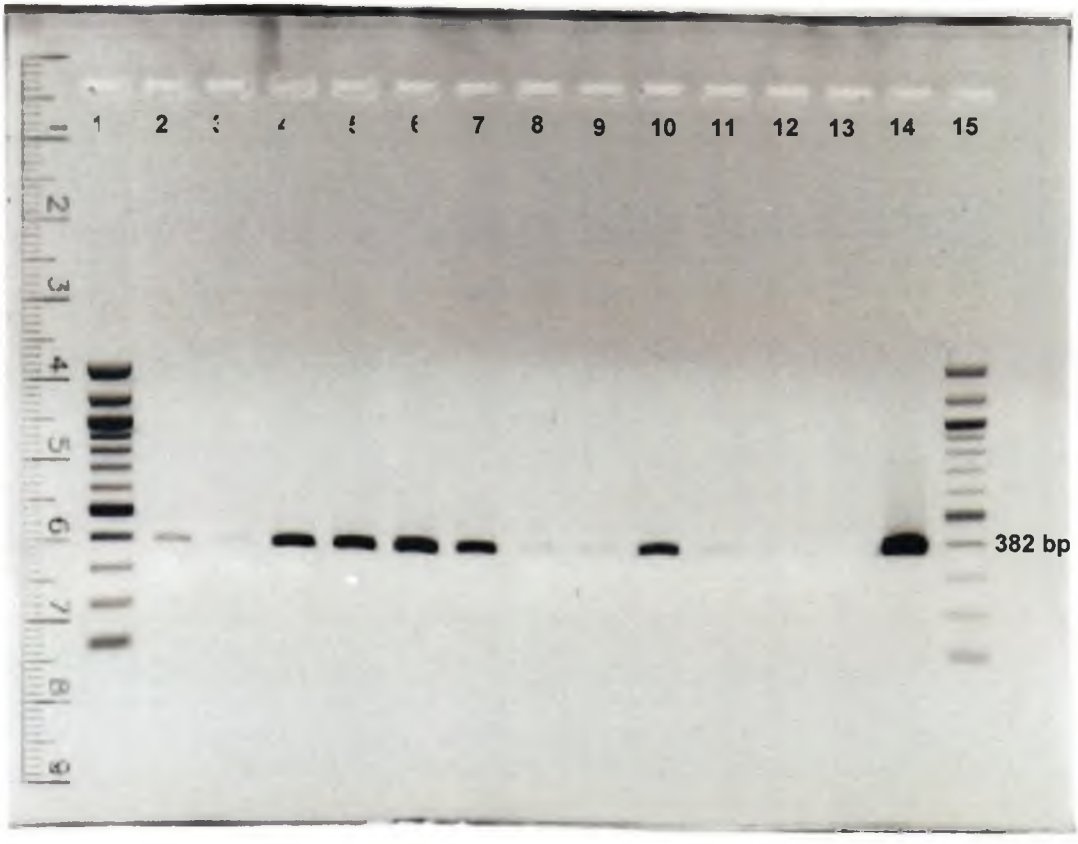

Figure 4.6 Agarose gel electrophoresis of PCR amplified products of *Mut11* gene by using primers series 2. Primers specific for UBQ3 promoter and *Mut11*gene were used to amplify a 382 bp fragment from genomic DNA of 10 individual transgenic plants. Lane 1& 15 = 100 bp markers; lanes 2-11 = genomic DNA from 10 individual sample of UH800 and UH 306; lane 12 = WT; lane 13 = water; lane 14 = *Mut1* plasmid. Lane 3 and 8 are virus infected transgenic plants, lane 1, 2, 4-7, and 9-10 are virus free transgenic plants. PCR products from lane 6 and lane 8 were sequenced.

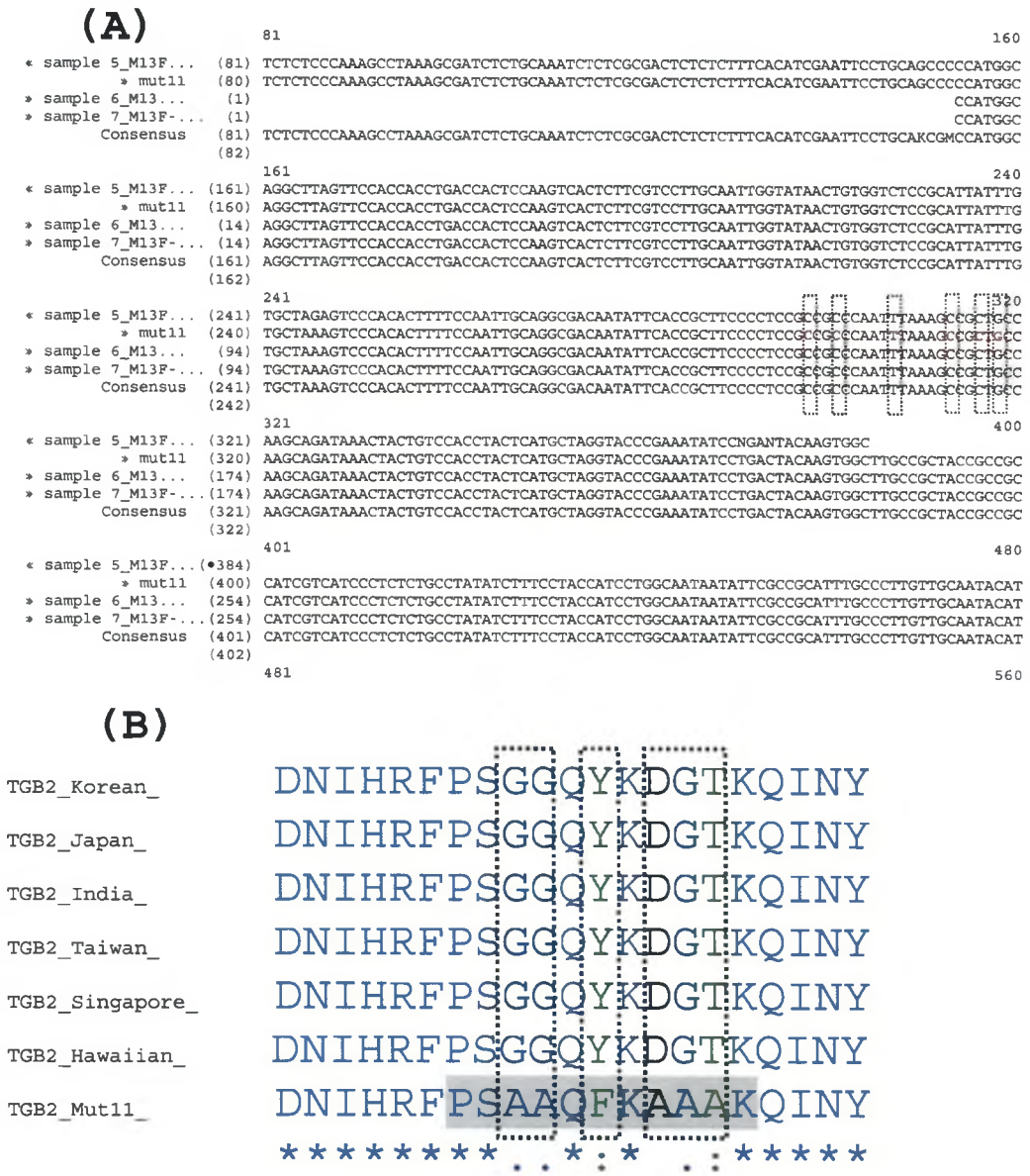

Figure 4.7 (A) Alignment for PCR fragment from sample 5 and 7 with *Mut11* template. The letter in the box indicated the six different bases changed from movement protein of CymMV Hawaiian strain wild type. (B) Alignment of amino acid sequences of the triple gene block 2(TGB2) of six strains of CymMV and the *Mut11* resistance gene, indicating the six amino acids different of *Mut11*.

4.5 DISCUSSION

This study produced eight individual plants of commercial *Dendrobium* x Jaquelyn Thomas 'Uniwai Mist' (UH800) resistant to CymMV virus. Five of eight lines lacked any selectable marker gene, making them particularly valuable and safer for field tests in the future. The MP strategy thus was effective in this orchid. Several studies have stated that the movement protein (MP) plays a key role in cell-to-cell spread of virus infection (Doem *et al.*, 1987; Doem *et al.*, 1992). Doem *et al.* (1990) indicated that the role of the MP of tobacco mosaic virus is to facilitate the cell-to-cell spread of viral progeny during infection. Viruses or their genome use movement proteins to move from cell to cell via plasmodesmata. The production of a mutated movement protein in transgenic plants interferes with this process by competing with the wild-type protein for binding sites on the plasmodesmata. Resistance to TMV and other viruses has been recorded in tobacco plants expressing a mutated version of the TMV movement protein gene. The amount of virus at infection sites was not decreased but their diffusion through the plant was impaired (Beck *et al.*, 1994; Cooper *et al.*, 1995). Movement protein-mediated virus resistance is generally efficient and unspecific. Karger *et al.* (2003) elucidated that the function of Thr^{104} for TMV cell-to-cell movement, by using a modified MP. The mutant viruses referred to as $Thr^{104}A$ (substitute Thr^{104} by Ala) and $Thr^{104}D$ (substitute Thr^{104} by Asp) were compared by inoculation of indicator plants reacting to TMV infection by production of local lesions or systemic symptoms. The result indicated that the only tiny local lesions were produced by $Thr^{104}D$ mutant, suggesting that the Thr to Asp substitution at position 104 strongly inhibited virus cell-to-

cell movement (Karger *et al.*, 2003). The Ob strain of tobamovirus movement protein (MP) fused with the gene encoding green fluorescent protein (GFP) from the jelly fish *Aequorea victoria,* was used to study transient movement protein expression. The results indicated that the expression of a fluorescent fusion protein (MP: : GFP) was fully active in mediating the cell-to-cell spread of the Ob-virus (Bernard *et al.*, 1996).

Our experiment used a dysfunctional movement protein with a site-specific mutation (*Mut11*) transformed into UH800 and UH306 to evaluate the genetic engineering, expression and resistance to CymMV. Multiple studies had previously indicated that mutants of the movement protein provide much greater levels of resistance than wild type MP according to the mutant MP will interfere with the wild type MP that is responsible for the modification of plasmodesmata (Beck *et al.*, 1994). From detailed studies on viruses like TMV, it was shown that some strains of virus were unable to move from cell to cell and produced very limited disease symptoms. The reason these viruses could not move was because they have a defective movement protein, one that prevented the plasmodesmata from enlarging. When this defective movement protein was expressed in transgenic plants, it was found to provide resistance against viruses, not by preventing the infection of plant cells but by preventing the spread of virus from cell to cell (Konduru *et al.*, 2003; Kotlizky *et al.*, 2001; Cooper *et al.*, 1995; Fenczik *et al.*, 1995; Beck *et al.*, 1994). In our study, we observed that transgenic *Dendrobium* orchids that expressed the *Mut11* transgene according to RT-PCR analysis result could not confer complete protection against CymMV infection as shown by TBIA analysis. It did, however delay the systemic infection of CymMV compared to wild type control that

became quickly infected after the first inoculation with a 1: 1000 dilution of CymMV. One plant (UH306-7II-D) became infected systemically based on TBIA analysis after the second inoculation with 1: 1000 dilution of CymMV while, another plant (UH800-1-B) became infected systemically only after the third inoculation with 1: 1000 dilution of CymMV. It is possible that in these plants, contain insufficient copy number of the transgene to provide transgenic *Dendrobium* orchid's resistance to CymMV. The relationship between copy number of transgene and virus resistance has been studied previously in tobacco (Goodwin *et al.* 1996). The experiment indicated that transgenic lines with one or two copies of tobacco etch virus coat protein (TEV CP) gene insertions showed only inducible resistance, whereas transgenic lines with three or more copy of TEV CP gene insertions were highly resistance to TEV.

The eight plants that we obtained showed no signs of infection from CymMV after three inoculations with 1: 1000 dilution of CymMV over 12 months period. All eight virus-free plants showed positive results from PCR analysis, but negative results from RT-PCR analysis. These results lead us to hypothesize that a post-transcriptional gene silencing (PTGS) mechanism might be involved in conferring resistance to CymMV. The PTGS process involves homology-dependent pairing between transgenes or between transgene and viral or transgene mRNA (Liao *et al.* 2004). In this system, a viral vector carrying a copy of the gene to be silenced is introduced into the cell. The cellular machinery recognizes the viral threat and releases a protective defense to destroy not only the viral genes but also any extra-gene being carried by the viral vector, affecting any native or transgenic homologous transcripts (Ruiz *et al.*, 1998; Waterhouse

et al., 2001). It has been demonstrated that the gene silencing mechanism is conserved and that the phenomenon can function in monocots, including several Graminae species, bulbous monocots such as lily, and in orchids (Iyer *et al.* 2000). Coat protein transgenes may protect transgenic plants by way of PTGS (Chiang *et al.* 2001; Liao *et al.* 2004).

Several mechanisms have been proposed to explain how PTGS results in viral resistance in transgenic plants (Waterhouse *et al.*, 1998; Baulcombe, 2002; Mlotshwa *et al.*, 2002; Benedito *et al.*, 2004). When PTGS is active in cells, small sequence-specific sense and anti-sense RNAs of 21 to 23 nucleotide can be detected (Hamilton *et al.*, 2002). These small interfering RNAs (siRNA) are not only indicative of PTGS, but also play a key role in the PTGS mechanism (Waterhouse *et al.*, 2001). Analysis using nuclear run-on and siRNA detection in transgenic *Dendrobium* plants should be conducted in the future to confirm this hypothesis.

Selection is the most crucial step in plant transformation since this is the key to separate the transformed plants from non-transformed plants. Our experiment suggests that a 31.5% survival rate on non-bombarded control UH800 plants will allow a good selection pressure to kill most of the non-transformed UH800 plants and is not toxic enough to kill the transformed UH800 plants. However, this selection does not eliminate all non-transformed UH800 plants. We obtained 16% of non-transformed plants, escaped from this selection regime. High levels of antibiotic for short period of time have the potential to be toxic, killing the transformed plants along with the non-transformed ones.

Although it is difficult to recommend a single selection procedure for UH306 and UH800, our percentage survival data and PCR results can be used as a guideline to avoid

antibiotic concentration that are too low or too high. For UH800, two weeks in a liquid medium containing a hygromycin B concentration of 12.5 $mg\,L^{-1}$ is a good selection regime for PLBs of UH800 bombarded with pSAN154. Whereas two weeks in a liquid medium containing a hygromycin B concentration of 20.0 $mg\,L^{-1}$ is a suitable selection regime for PLBs of UH306 bombarded with pSAN154.

Figure 4.8 Acclimatized transformed *D.* x Jaquelyn Thomas 'Uniwai Mist'(UH800) and *D.* x Jaq – Hawaii 'Uniwai Pearl'(UH306) growing in screened insect proof boxes in University of Hawaii Greenhouse facility. Surrounding pots contained wild-type seedlings.

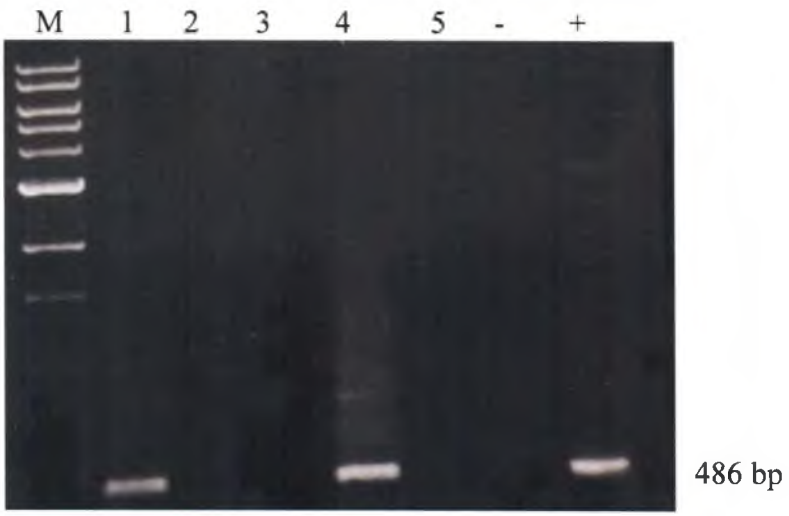

Figure 4.9 Agarose gel electrophoresis of RT-PCR products of amplified cDNA from transgenic *Dendrobium* UH800-1-B (lane1) and UH306-7II-D (lane 4) indicated to contain the Mut11 gene by PCR. Primers used for amplification were specific to a 486 bp fragment of Mut11 gene. Lanes 1-5 = cDNA from individual plants; lane m = molecular weight marker; lane - = negative control, cDNA from virus free WT; lane + = plasmid DNA from Mut11 plasmid.

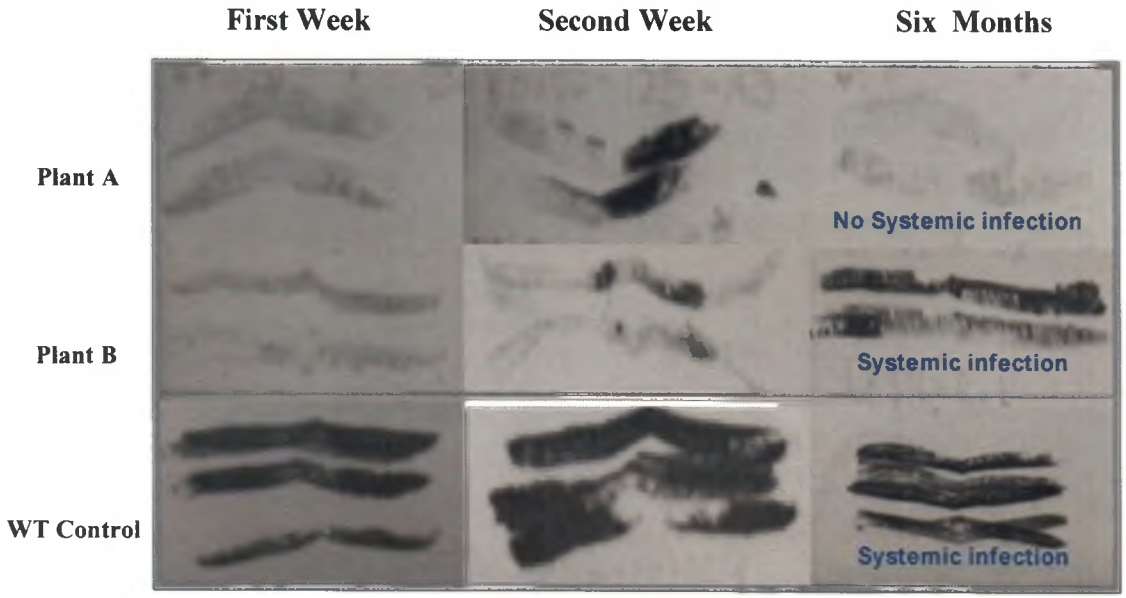

Figure 4.10 Parts of result from Leaf Tissue Blot Immunoassay. Dark regions indicate virus. Transgenic plant A has no systemic infection compared to transgenic plant B or wild type (WT) control.

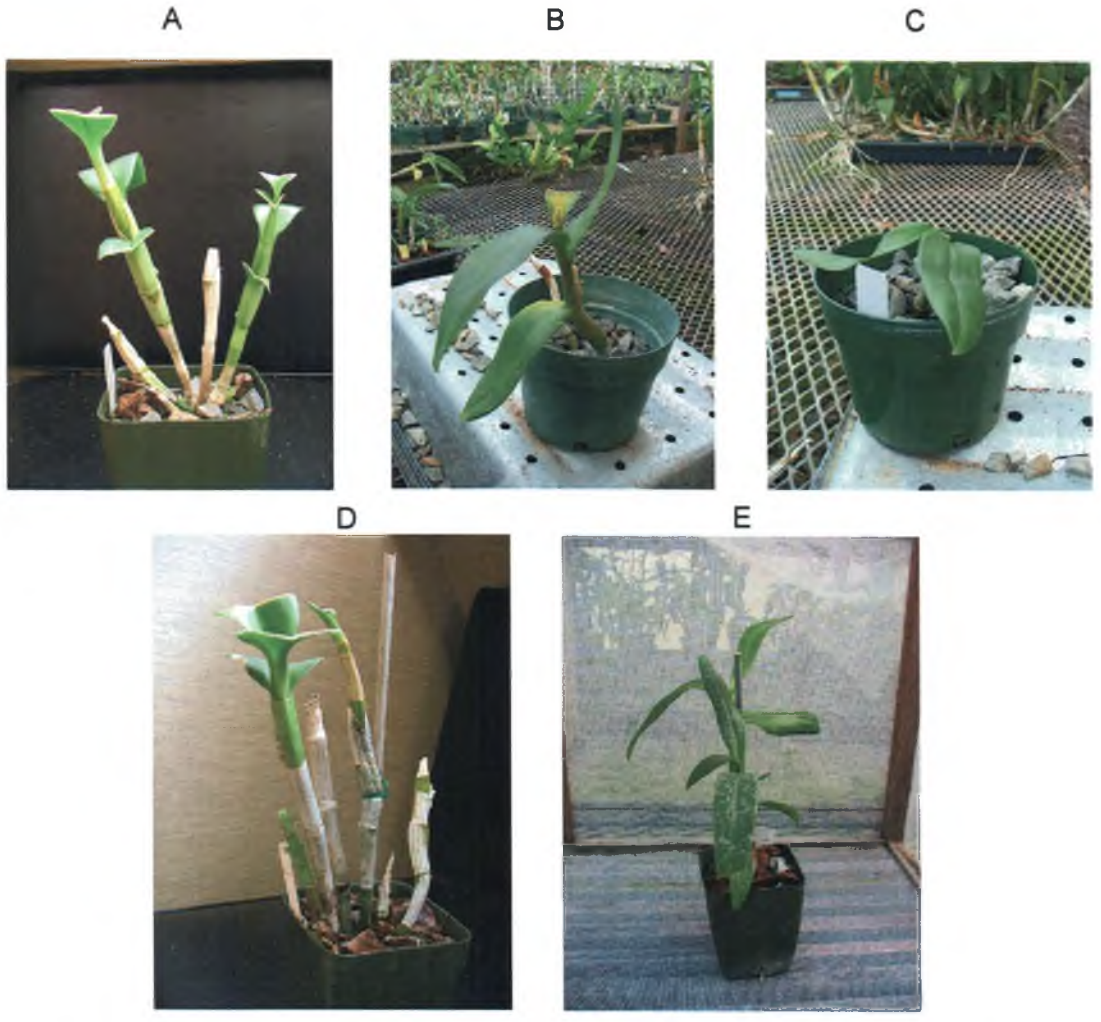

Figure 4.11 All five individual viral free transgenic *Dendrobium* orchids are waiting for clonal propagation

A = UH800II-15A-2

B = UH800II-15A-10

C = UH800II-15A-1

D = UH800II-15A-4

E = UH800-12B-A4

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